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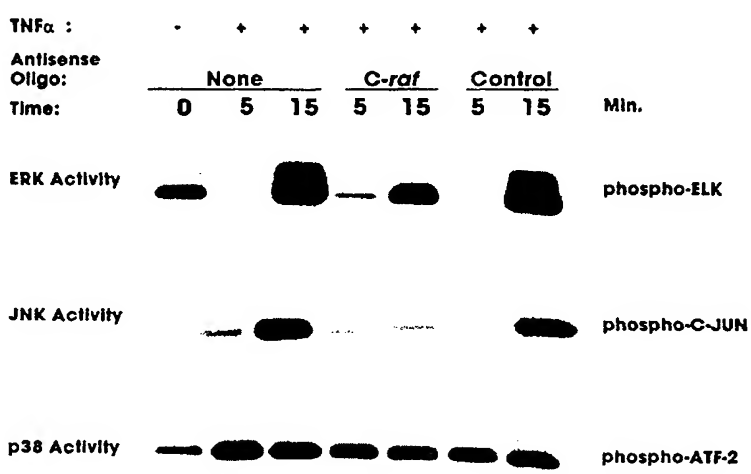

(57) Abstract

Methods are provided for inhibiting the expression of cell adhesion molecules using inhibitors of signaling molecules involved in human TNF–α signaling. These inhibitors include monoclonal antibodies, peptide fragments, small molecule inhibitors, and, preferably, antisense oligonucleotides. Methods for treatment of diseases, particularly inflammatory and immune diseases, associated with overexpression of cell adhesion molecules are provided.

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METHODS OF MODULATING TUMOR NECROSIS FACTOR α-INDUCED EXPRESSION OF CELL ADHESION MOLECULES

FIELD OF THE INVENTION

The present invention relates to the modulation of expression of cell adhesion molecules. In particular, herein are provided methods of inhibiting cell adhesion molecule gene expression through specific inhibitors involved in TNF-α signaling. Methods are also provided for treating inflammatory and immune diseases associated with altered expression of cell adhesion molecules.

BACKGROUND OF THE INVENTION

Cytokines represent a diverse group of regulatory proteins with numerous biological functions including cell differentiation, cell growth, and cytotoxity. Inflammatory cytokines such as Tumor Necrosis Factor alpha and (TNF-α)and IL-1 (interleukin-1) have been shown to play pivotal roles in immune and inflammatory responses (McIntyre, T.M., et al., Thromb. Haemos. 1997, 78, 302-305). One of the most important effector functions of these cytokines is their ability to induce profound changes in the vascular endothelium (Introna, M. and Mantovani, A., Art. Thromb. and Vasc. Biol. 1997, 17, 423-428). Central to the process of inflammation is the induction of cell adhesion molecules on the endothelial cell surface, contributing significantly to the adherence and recruitment of circulating leukocytes to inflammatory sites. Upon exposure to TNF-α or IL-1, which are produced in response to injury or infection, cytokine receptors on endothelial cells activate a variety of intracellular signaling molecules. These signaling events result in the activation of specific transcription factors such as NF-kB and upregulate the expression of E-selectin, ICAM-1, VCAM-1, and other cell adhesion molecules (McIntyre, T.M., et al., Thromb. Haemos. 997, 78, 302-305; Introna, M. and Mantovani, A., Art. Thromb. and Vasc. Biol.

1997, 17, 423-428; Mantovani, A., et al., Thromb. Haemos. 1997, 78, 406-414). E-selectin has been shown to mediate the initial attachment and rolling of leukocytes along the vessel wall, whereas ICAM-1 and VCAM-1 are involved in the firm adhesion of leukocytes to the vessel wall and their transmigration through the vessel wall. E-selectin is rapidly and transiently induced by cytokines with peak expression occurring approximately 4-6 hours after exposure and returning to basal levels approximately 24 hours post exposure. In contrast, induction of ICAM-1 and VCAM-1 by cytokines is slower and persists for 24 hours or longer (Mantovani, A., et al., Thromb. Haemos. 1997, 78, 406-414; Dunon, D., et al., Curr. Opin. Cell Bio, 1996, 8, 714-723; Bischoff, J., Cell Adhes. and Angiog., 1997, 99, 373-376).

Responses to TNF-α are mediated through interactions with two distinct membrane receptors, termed TNFRI (TNF-α receptor I) and TNFRII (TNF-α receptor II). Two distinct families of adaptor proteins associated with TNF-α receptors have been identified. The death domain-containing proteins (e.g., TRADD) appear to couple the receptors to programmed cell death (Fiers, W., et al., J. Inflam., 1996, 47, 67-75; Wallach, D., et al., FEBS Lett., 1997, 410, 96-106; Hsu, H., et al., Cell, 1996, 84, 299-308), whereas the TRAF (TNF receptor associated factor) domain-containing proteins link the receptors to activation of specific transcription factors (Hsu, H., et al., Cell, 1996, 84, 299-308; Baeuerle, P.A., Curr. Biol., 1998, 8, R19-R22). Among the six members of the TRAF family that have been identified so far, TRAF2 has been reported to be important for TNF-α-mediated activation of JNK (c-Jun N-terminal kinase), as well as two major transcription factors, NF-kB (nuclear factor-kB) and AP-1 (activator protein 1) (Hsu, H., et al., Cell, 1996, 84, 299-308; Baeuerle, P.A., Curr. Biol., 1998, 8, R19-R22; Natoli, G., et al., J. Biol. Chem., 1997, 272, 26079-26082; Liu, Z.G.,

et al., Cell, 1996, 87, 565-576; Song, H.Y., et al., Proc. Natl. Acad. Sci. USA, 1997, 94, 9792-9796). Both transcription factors play pivotal roles in the regulation of multiple genes including those involved in immune and inflammatory responses. AP-1 is activated by various MAPKs (mitogen-activated protein kinase) including ERK (extracellular-signal-regulated kinase), JNK and p38 MAPK (Fiers, W., et al., J. Inflam., 1996, 47, 67-75; Eder, J., TIPS, 1997, 18, 319-322). NF-kB is constitutively present in the cytosol of endothelial cells and kept inactive by association with inhibitory IkB family proteins. Upon exposure to TNF-α, IKK (IkB kinase) phosphorylates IkB and initiates its ubiquitination and subsequent degradation. The released NF-kB translocates to the nucleus and participates in transcriptional activation (Collins, T., et al., FASEB J., 1995, 9, 899-909; Stancovski, I., and Baltimore, D., Cell, 1997, 91, 299-302).

Other signaling molecules, including MEKK1, pp90rsk (ribosomal S6 protein kinase), *ras*, and *raf*, have been implicated in the activation of NF-kB (Schulze-Osthoff, K., et al., Immunobiol., 1997, 198, 35-49). *ras* family members (Ha-*ras*, Ki-*ras*, N-*ras*) are GTP-binding proteins that act as major mediators in the regulation of cell proliferation and differentiation in response to a variety of extracellular stimuli including TNF-α (Bos, J.L., Biochem. Biophys. Acta, 1997, 1333, M19-M31). *ras* proteins have been shown to activate both the *raf*/MEK/ERK pathway as well as MEKK/JNKK/JNK pathway (Bos, J.L., Biochem. Biophys. Acta, 1997, 1333, M19-M31; Marais, R., and Marshall, C.J., Cancer Surveys, 1996, 27, 101-125; Adler, V., et al., J. Biol. Chem., 1996, 271, 23304-23309; Faris, M., et al., J. Biol. Chem., 1996, 271, 27366-27373; Terada, K., et al., J. Biol. Chem., 1997, 272, 4544-4548). raf family members (A-, B-, *c-raf*) are serine/threonine protein kinases that transmit signals from cell surface receptors to a variety

of intracellular effectors including the MAPK pathways (Marais, R., and Marshall, C.J., Cancer Surveys, 1996, 27, 101-125; Daum, G., et al., TIBS, 1994, 19, 474-480). Besides *ras*, a variety of protein kinases including Src family kinases and PKC (protein kinase C) can potentiate *raf* activity (Marais, R., et al., J. Biol. Chem., 1997, 272, 4378-4383; Ueffing, M., et al., Oncogene, 1997, 15, 2921-2927). The major downstream effectors of *raf* are MEK/MKK1 (MAP kinase kinase 1) and MEK/MKK2 (MAP kinase kinase 2) which in turn phosphorylate and activate ERK1/2, and ultimately activate specific transcription factors (Marais, R., and Marshall, C.J., Cancer Surveys, 1996, 27, 101-125; Daum, G., et al., TIBS, 1994, 19, 474-480). Both *ras* and *raf* had been suggested to participate in the activation of NF-kB transcription factors (Schulze-Osthoff, K., et al., Immunobiol., 1997, 198, 35-49; Folgueira, L., et al., J. Virol., 1996, 70, 2332-2338; Koong, A.C., et al., Cancer Res., 1994, 54, 5273-5279; Bertrand, F., et al., J. Biol. Chem., 1995, 270, 24435-24441; Kanno, T., and Siebenlist, U., J. Immunol., 1996, 157, 5277-5283).

In many human diseases with an inflammatory component, the normal, homeostatic mechanisms which attenuate the inflammatory responses are defective, resulting in damage and destruction of normal tissue. For example, VCAM-1 may play a role in the metastasis of melanoma, and possibly other cancers. In addition, data have demonstrated that ICAM-1 is the cellular receptor for the major serotype of rhinovirus, which account for greater than 50% of common colds. (Staunton, et al., Cell, 1989, 56, 849-853; Greve et al., Cell, 1989, 56, 839-847).

Expression of ICAM-1 has also been associated with a variety of inflammatory skin disorders such as allergic contact dermatitis, fixed drug eruption, lichen planus, and psoriasis (Ho, et al., J. Am. Acad. Dermatol., 1990, 22, 64-68; Griffiths and Nickoloff, Am. J. Pathology, 1989,

135, 1045-1053; Lisby, et al., Br. J. Dermatol., 1989, 120, 479-484; Shiohara, et al., Arch. Dermatol., 1989, 125, 1371-1376). In addition, ICAM-1 expression has been detected in the synovium of patients with rheumatoid arthritis (Hale, et al., Arth. Rheum., 1989, 32, 22-30), pancreatic B-cells in diabetes (Campbell, et al., Proc. Natl. Acad. Sci. U.S.A., 1989, 86, 4282-4286), thyroid follicular cells in patients with Graves' disease (Weetman, et al., J. Endocrinol., 1989, 122, 185-191), and with renal and liver allograft rejection (Faull and Russ, Transplantation, 1989, 48, 226-230; Adams, et al., Lancet, 1989, 2, 1122-1125).

Inhibitors of ICAM-1, VCAM-1 and ELAM-1 expression would provide a novel therapeutic class of anti-inflammatory agents with activity towards a variety of inflammatory diseases or diseases with an inflammatory component such as asthma, rheumatoid arthritis, allograft rejections, inflammatory bowel disease, various dermatological conditions, and psoriasis. In addition, inhibitors of ICAM-1, VCAM-1, and ELAM-1 may also be effective in the treatment of colds due to rhinovirus infection, AIDS, Kaposi's sarcoma and some cancers and their metastasis. The use of neutralizing monoclonal antibodies against ICAM-1 in animal models provide evidence that such inhibitors if identified would have therapeutic benefit for asthma (Wegner, et al., Science, 1990, 247, 456-459), renal allografts (Cosimi, et al., J. Immunol., 1990, 144, 4604-4612), and cardiac allografts (Isobe, et al., Science, 1992, 255, 1125-1127). The use of a soluble form of ICAM-1 molecule was also effective in preventing rhinovirus infection of cells in culture (Marlin, et al., Nature, 1990, 344, 70-72).

Current agents which affect intercellular adhesion molecules include synthetic peptides, monoclonal antibodies, soluble forms of the adhesion molecules, and

antisense oligonucleotides. Antisense oligonucleotides to cell adhesion molecules are disclosed in US Patent Nos. 5,514,788 and 5,591,623, herein incorporated by reference. These have been directed against a single cell adhesion molecule. Additional agents are desired. Furthermore, a broader approach, targeting several adhesion molecules with a single agent may have several advantages, including economies of scale, broad spectrum utility, etc. Thus, an approach to target a molecule in the TNF-α signaling pathway may be a useful therapeutic treatment, providing a means to regulate multiple cell adhesion molecules with a single agent.

Inhibitors of molecules in TNF-α mediated signaling have been used to study the signal transduction pathways and suggest utility in the design of pharmacological agents. Inhibitors that have been used include DMSO (Essani, N.A., et al., Shock, 1997, 7, 90-96) against NF-kB, protein tyrosine kinase inhibitors (Adamson, P., et al., Cell Adhes. Commun.,1996, 3, 511-525; Pai, R., et al., J. Immunol., 1996, 156, 2571-2579), protein tyrosine kinase C inhibitors (Ballestas, M.E. and Benveniste, E.N., Glia, 1995, 14, 267-278), ubiquitin ligase inhibitors (Yaron, A., et al., EMBO J., 1997, 16, 6486-6494), and phospholipase A2 inhibitors (Thommesen, L., et al., J. Immunol., 1998, 161, 3421-3430). In addition, drugs that elevate cyclic AMP have been found to inhibit ELAM-1 and VCAM-1 (Pober, J.S., et al., J. Immunol., 1993, 150, 5114-5123).

Antisense oligonucleotides to *c-raf*, Ha-*ras* and JNK2 are known, but have not previously been shown to inhibit cell adhesion molecule expression. The relationship between these TNF-α signaling molecules and cell adhesion molecule expression has not been fully delineated. *c-raf* antisense oligonucleotides are disclosed in US Patent Nos. 5,563,255 and 5,656,612, herein incorporated by reference. Ha-ras antisense oligonucleotide are disclosed in US Patent

Nos. 5,576,208 and 5,582,986, herein incorporated by reference. JNK2 antisense oligonucleotides are disclosed by Bost, F., et al. (J. Biol. Chem. 1997, 272, 33422-33429). Inhibitors of the TNF-α signaling molecules, *c-raf*, Ha-*ras* and JNK2 have not been used to modulate expression of cell adhesion molecules and represent a novel approach.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a Western blot showing a time-course of the effects of *c-raf* antisense oligonucleotides on *c-raf* and a-*raf* protein levels.

FIG. 2 is a Western blot showing a time-course of the effects of Ha-*ras* antisense oligonucleotides on Ha-*ras* and Ki-*ras* protein levels.

FIG. 3 is a Western blot showing the effects of *c-raf* antisense oligonucleotides on TNF-α mediated ERK, JNK and p38 kinase activities. Phospho-substrate-specific antibodies were used to analyze kinase activities.

FIG. 4 is Northern blot showing the effects of JNK1 and JNK2 antisense oligonucleotides on TNF-α mediated JNK1 and JNK2 mRNA expression.

FIG. 5 is a Western blot showing the effects of JNK1 and JNK2 antisense oligonucleotides on TNF-α mediated JNK1 and JNK2 kinase activity. Phospho-substrate-specific antibodies were used to analyze kinase activities.

BRIEF DESCRIPTION OF THE INVENTION

The present invention describes a method of modulating cell adhesion molecule expression within a cell comprising treating said cell with a specific inhibitor of one of the Tumor Necrosis Factor alpha (TNF-α) signaling molecules, Ha-ras, *c-raf* or JNK2. In one embodiment, the specific inhibitor is an antisense oligonucleotide capable of hybridizing to Ha-ras, *c-raf* or JNK2. Also provided are methods of treating an inflammatory or immune disease or

condition associated with altered expression of a cell adhesion molecule comprising administering a specific inhibitor of one of the TNF-α signaling molecules, Ha-*ras*, *c-raf* or JNK2.

DETAILED DESCRIPTION OF THE INVENTION

The present invention employs specific inhibitors of Ha-*ras*, *c-raf* and JNK2, members of the TNF-α signaling pathway, to modulate cell adhesion molecule expression. These inhibitors can include monoclonal antibodies, peptide fragments, small molecule inhibitors and antisense compounds. In a preferred embodiment, antisense compounds, particularly oligonucleotides, are used to modulate the function of nucleic acid molecules encoding Ha-*ras*, *c-raf* or JNK2, modulating the amount of protein produced and ultimately modulating the expression of cell adhesion molecules. This is accomplished by providing oligonucleotides which specifically hybridize with nucleic acids, preferably mRNA, encoding Ha-*ras*, *c-raf* or JNK2.

This relationship between an antisense compound such as an oligonucleotide and its complementary nucleic acid target, to which it hybridizes, is commonly referred to as "antisense". "Targeting" an oligonucleotide to a chosen nucleic acid target, in the context of this invention, is a multistep process. The process usually begins with identifying a nucleic acid sequence whose function is to be modulated. This may be, as examples, a cellular gene (or mRNA made from the gene) whose expression is associated with a particular disease state, or a foreign nucleic acid from an infectious agent. In the present invention, the targets are nucleic acids encoding Ha-*ras*, *c-raf* or JNK2; in other words, a gene encoding Ha-*ras*, *c-raf* or JNK2, or mRNA expressed from the Ha-*ras*, *c-raf* or JNK2 gene. mRNA which encodes Ha-*ras*, *c-raf* or JNK2 is presently the preferred target. The targeting process also includes

determination of a site or sites within the nucleic acid sequence for the antisense interaction to occur such that modulation of gene expression will result.

In accordance with this invention, persons of ordinary skill in the art will understand that messenger RNA includes not only the information to encode a protein using the three letter genetic code, but also associated ribonucleotides which form a region known to such persons as the 5'-untranslated region, the 3'-untranslated region, the 5' cap region and intron/exon junction ribonucleotides. Thus, oligonucleotides may be formulated in accordance with this invention which are targeted wholly or in part to these associated ribonucleotides as well as to the informational ribonucleotides. The oligonucleotide may therefore be specifically hybridizable with a transcription initiation site region, a translation initiation codon region, a 5' cap region, an intron/exon junction, coding sequences, a translation termination codon region or sequences in the 5'- or 3'-untranslated region. Since, as is known in the art, the translation initiation codon is typically 5'-AUG (in transcribed mRNA molecules; 5'-ATG in the corresponding DNA molecule), the translation initiation codon is also referred to as the "AUG codon," the "start codon" or the "AUG start codon." A minority of genes have a translation initiation codon having the RNA sequence 5'-GUG, 5'-UUG or 5'-CUG, and 5'-AUA, 5'-ACG and 5'-CUG have been shown to function *in vivo*. Thus, the terms "translation initiation codon" and "start codon" can encompass many codon sequences, even though the initiator amino acid in each instance is typically methionine (in eukaryotes) or formylmethionine (prokaryotes). It is also known in the art that eukaryotic and prokaryotic genes may have two or more alternative start codons, any one of which may be preferentially utilized for translation initiation in a particular cell type or tissue, or under a particular

set of conditions. In the context of the invention, "start codon" and "translation initiation codon" refer to the codon or codons that are used *in vivo* to initiate translation of an mRNA molecule transcribed from a gene encoding Ha-*ras*, *c-raf* or JNK2, regardless of the sequence(s) of such codons. It is also known in the art that a translation termination codon (or "stop codon") of a gene may have one of three sequences, *i.e.*, 5'-UAA, 5'-UAG and 5'-UGA (the corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA, respectively). The terms "start codon region," "AUG region" and "translation initiation codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (*i.e.*, 5' or 3') from a translation initiation codon. This region is a preferred target region. Similarly, the terms "stop codon region" and "translation termination codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (*i.e.*, 5' or 3') from a translation termination codon. This region is a preferred target region. The open reading frame (ORF) or "coding region," which is known in the art to refer to the region between the translation initiation codon and the translation termination codon, is also a region which may be targeted effectively. Other preferred target regions include the 5' untranslated region (5'UTR), known in the art to refer to the portion of an mRNA in the 5' direction from the translation initiation codon, and thus including nucleotides between the 5' cap site and the translation initiation codon of an mRNA or corresponding nucleotides on the gene and the 3' untranslated region (3'UTR), known in the art to refer to the portion of an mRNA in the 3' direction from the translation termination codon, and thus including nucleotides between the translation termination codon and 3' end of an mRNA or

corresponding nucleotides on the gene. The 5' cap of an mRNA comprises an N7-methylated guanosine residue joined to the 5'-most residue of the mRNA via a 5'-5' triphosphate linkage. The 5' cap region of an mRNA is considered to include the 5' cap structure itself as well as the first 50 nucleotides adjacent to the cap. The 5' cap region may also be a preferred target region.

Although some eukaryotic mRNA transcripts are directly translated, many contain one or more regions, known as "introns," which are excised from a pre-mRNA transcript to yield one or more mature mRNA. The remaining (and therefore translated) regions are known as "exons" and are spliced together to form a continuous mRNA sequence. mRNA splice sites, *i.e.*, exon-exon or intron-exon junctions, may also be preferred target regions, and are particularly useful in situations where aberrant splicing is implicated in disease, or where an overproduction of a particular mRNA splice product is implicated in disease. Aberrant fusion junctions due to rearrangements or deletions are also preferred targets. Targeting particular exons in alternatively spliced mRNAs may also be preferred. It has also been found that introns can also be effective, and therefore preferred, target regions for antisense compounds targeted, for example, to DNA or pre-mRNA.

Once the target site or sites have been identified, oligonucleotides are chosen which are sufficiently complementary to the target, *i.e.*, hybridize sufficiently well and with sufficient specificity, to give the desired modulation.

"Hybridization", in the context of this invention, means hydrogen bonding, also known as Watson-Crick base pairing, between complementary bases, usually on opposite nucleic acid strands or two regions of a nucleic acid strand. Guanine and cytosine are examples of complementary bases which are known to form three hydrogen bonds between

them. Adenine and thymine are examples of complementary bases which form two hydrogen bonds between them.

"Specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity such that stable and specific binding occurs between the DNA or RNA target and the oligonucleotide.

It is understood that an oligonucleotide need not be 100% complementary to its target nucleic acid sequence to be specifically hybridizable. An oligonucleotide is specifically hybridizable when binding of the oligonucleotide to the target interferes with the normal function of the target molecule to cause a loss of utility, and there is a sufficient degree of complementarity to avoid non-specific binding of the oligonucleotide to non-target sequences under conditions in which specific binding is desired, *i.e.*, under physiological conditions in the case of *in vivo* assays or therapeutic treatment and, in the case of *in vitro* assays, under conditions in which the assays are conducted.

Hybridization of antisense oligonucleotides with mRNA interferes with one or more of the normal functions of mRNA. The functions of mRNA to be interfered with include all vital functions such as, for example, translocation of the RNA to the site of protein translation, translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, and catalytic activity which may be engaged in by the RNA. Binding of specific protein(s) to the RNA may also be interfered with by antisense oligonucleotide hybridization to the RNA.

The overall effect of interference with mRNA function is modulation of expression of *c-raf*, Ha-*ras* or JNK2 and, in the context of this invention, ultimately modulation of cellular adhesion molecule expression. In the context of this invention "modulation" means either inhibition or

stimulation; *i.e.*, either a decrease or increase in expression. This modulation can be measured in ways which are routine in the art, for example by Northern blot assay of mRNA expression, or reverse transcriptase PCR, as taught in the examples of the instant application or by Western blot or ELISA assay of protein expression, or by an immunoprecipitation assay of protein expression. Effects on cell proliferation or tumor cell growth can also be measured, as taught in the examples of the instant application. Inhibition is presently preferred.

The oligonucleotides of this invention can be used in diagnostics, therapeutics, prophylaxis, and as research reagents and in kits. Since the oligonucleotides of this invention hybridize to nucleic acids encoding Ha-*ras*, *c-raf* or JNK2, sandwich, colorimetric and other assays can easily be constructed to exploit this fact. Provision of means for detecting hybridization of oligonucleotide with the Ha-*ras*, *c-raf* or JNK2 gene or mRNA can routinely be accomplished. Such provision may include enzyme conjugation, radiolabelling or any other suitable detection systems. Kits for detecting the presence or absence of Ha-*ras*, *c-raf* or JNK2 may also be prepared.

The present invention is also suitable for diagnosing abnormal inflammatory states in tissue or other samples from patients suspected of having an inflammatory disease such as rheumatoid arthritis. The ability of the oligonucleotides of the present invention to inhibit inflammatory processes may be employed to diagnose such states. A number of assays may be formulated employing the present invention, which assays will commonly comprise contacting a tissue sample with an oligonucleotide of the invention under conditions selected to permit detection and, usually, quantitation of such inhibition. In the context of this invention, to "contact" tissues or cells with an oligonucleotide or oligonucleotides means to add

the oligonucleotide(s), usually in a liquid carrier, to a cell suspension or tissue sample, either *in vitro* or *ex vivo*, or to administer the oligonucleotide(s) to cells or tissues within an animal.

The oligonucleotides of this invention may also be used for research purposes. For example, the function of a specific gene product in a signaling pathway may be investigated using specific oligonucleotides. Thus, the specific hybridization exhibited by the oligonucleotides may be used for assays, purifications, cellular product preparations and in other methodologies which may be appreciated by persons of ordinary skill in the art.

In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid or deoxyribonucleic acid. This term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent intersugar (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced binding to target and increased stability in the presence of nucleases.

The antisense compounds in accordance with this invention preferably comprise from about 5 to about 50 nucleobases. Particularly preferred are antisense oligonucleotides comprising from about 8 to about 30 nucleobases (*i.e.* from about 8 to about 30 linked nucleosides). As is known in the art, a nucleoside is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar

portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2′, 3′ or 5′ hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn the respective ends of this linear polymeric structure can be further joined to form a circular structure, however, open linear structures are generally preferred. Within the oligonucleotide structure, the phosphate groups are commonly referred to as forming the internucleoside backbone of the oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3′ to 5′ phosphodiester linkage.

Specific examples of preferred antisense compounds useful in this invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

Preferred modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3′-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3′-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3′-5′ linkages, 2′-5′ linked analogs of these, and those having inverted polarity wherein the adjacent pairs

of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free acid forms are also included.

Representative United States patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S.: 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; and 5,625,050.

Preferred modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH_2 component parts.

Representative United States patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S.: 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; and 5,677,439.

In other preferred oligonucleotide mimetics, both the sugar and the internucleoside linkage, *i.e.*, the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA compounds include, but are not limited to, U.S.: 5,539,082; 5,714,331; and 5,719,262. Further teaching of PNA compounds can be found in Nielsen et al. (Science, 1991, 254, 1497-1500).

Most preferred embodiments of the invention are oligonucleotides with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular $-CH_2-NH-O-CH_2-$, $-CH_2-N(CH_3)-O-CH_2-$ [known as a methylene (methylimino) or MMI backbone], $-CH_2-O-N(CH_3)-CH_2-$, $-CH_2-N(CH_3)-N(CH_3)-CH_2-$ and $-O-N(CH_3)-CH_2-CH_2-$ [wherein the native phosphodiester backbone is represented as $-O-P-O-CH_2-$] of the above referenced U.S. patent 5,489,677, and the amide backbones of the above referenced U.S. patent 5,602,240. Also preferred are oligonucleotides having morpholino backbone structures of the above-referenced U.S. patent 5,034,506.

Modified oligonucleotides may also contain one or more substituted sugar moieties. Preferred oligonucleotides comprise one of the following at the 2' position: OH; F; O-, S-, or N-alkyl, O-alkyl-O-alkyl, O-, S-, or N-alkenyl, or O-, S- or N-alkynyl, wherein the alkyl, alkenyl and

alkynyl may be substituted or unsubstituted C_1 to C_{10} alkyl or C_2 to C_{10} alkenyl and alkynyl. Particularly preferred are $O[(CH_2)_nO]_mCH_3$, $O(CH_2)_nOCH_3$, $O(CH_2)_2ON(CH_3)_2$, $O(CH_2)_nNH_2$, $O(CH_2)_nCH_3$, $O(CH_2)_nONH_2$, and $O(CH_2)_nON[(CH_2)_nCH_3)]_2$, where n and m are from 1 to about 10. Other preferred oligonucleotides comprise one of the following at the 2′ position: C_1 to C_{10} lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3, OCN, Cl, Br, CN, CF_3, OCF_3, $SOCH_3$, SO_2CH_3, ONO_2, NO_2, N_3, NH_2, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy (2'-O-$CH_2CH_2OCH_3$, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin et al., Helv. Chim. Acta, 1995, 78, 486-504) *i.e.*, an alkoxyalkoxy group. A further preferred modification includes 2'-dimethylaminooxyethoxy, *i.e.*, a $O(CH_2)_2ON(CH_3)_2$ group, also known as 2'-DMAOE, as described in examples hereinbelow.

Other preferred modifications include 2'-methoxy (2'-O-CH_3), 2'-aminopropoxy (2'-$OCH_2CH_2CH_2NH_2$) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2′-5′ linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugars structures include, but are not limited to, U.S.: 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811;

5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,0531 5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920.

Oligonucleotides may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C or m5c), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further nucleobases include those disclosed in United States Patent No. 3,687,808, those disclosed in the Concise Encyclopedia Of Polymer Science And Engineering, 1990, pages 858-859, Kroschwitz, J.I., ed. John Wiley & Sons, those disclosed by Englisch et al. (Angewandte Chemie, International Edition, 1991, 30, 613-722), and those disclosed by Sanghvi, Y.S., Chapter 15, Antisense Research and Applications, 1993, pages 289-302, Crooke, S.T. and Lebleu, B., ed., CRC Press. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-

aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C (Sanghvi, Y.S., Crooke, S.T. and Lebleu, B., eds., Antisense Research and Applications, 1993, CRC Press, Boca Raton, pages 276-278) and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

Representative United States patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. 3,687,808, as well as U.S.: 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; and 5,681,941.

Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger et al., Proc. Natl. Acad. Sci. USA, 1989, 86, 6553-6556), cholic acid (Manoharan et al., Bioorg. Med. Chem. Lett., 1994, 4, 1053-1059), a thioether, e.g., hexyl-*S*-tritylthiol (Manoharan et al., Ann. N.Y. Acad. Sci., 1992, 660, 306-309; Manoharan et al., Bioorg. Med. Chem. Let., 1993, 3, 2765-2770), a thiocholesterol (Oberhauser et al., Nucl. Acids Res., 1992, 20, 533-538), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras et al., EMBO J., 1991, 10, 1111-1118; Kabanov et al., FEBS Lett., 1990, 259, 327-330; Svinarchuk et al., Biochimie, 1993, 75, 49-54), a phospholipid, e.g., di-hexadecyl-*rac*-glycerol or triethylammonium 1,2-di-O-hexadecyl-*rac*-glycero-3-H-phosphonate (Manoharan et al.,

Tetrahedron Lett., 1995, 36, 3651-3654; Shea et al., Nucl. Acids Res., 1990, 18, 3777-3783), a polyamine or a polyethylene glycol chain (Manoharan et al., Nucleosides & Nucleotides, 1995, 14, 969-973), or adamantane acetic acid (Manoharan et al., Tetrahedron Lett., 1995, 36, 3651-3654), a palmityl moiety (Mishra et al., Biochim. Biophys. Acta., 1995, 1264, 229-237), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke et al., J. Pharmacol. Exp. Ther., 1996, 277, 923-937).

Representative United States patents that teach the preparation of such oligonucleotide conjugates include, but are not limited to, U.S.: 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717, 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241, 5,391,723; 5,416,203, 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941.

The present invention also includes oligonucleotides which are chimeric oligonucleotides. "Chimeric" oligonucleotides or "chimeras," in the context of this invention, are oligonucleotides which contain two or more chemically distinct regions, each made up of at least one nucleotide. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the oligonucleotide may serve as a

substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of antisense inhibition of gene expression. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art. This RNAse H-mediated cleavage of the RNA target is distinct from the use of ribozymes to cleave nucleic acids. Ribozymes are not comprehended by the present invention.

Examples of chimeric oligonucleotides include but are not limited to "gapmers," in which three distinct regions are present, normally with a central region flanked by two regions which are chemically equivalent to each other but distinct from the gap. A preferred example of a gapmer is an oligonucleotide in which a central portion (the "gap") of the oligonucleotide serves as a substrate for RNase H and is preferably composed of 2'-deoxynucleotides, while the flanking portions (the 5' and 3' "wings") are modified to have greater affinity for the target RNA molecule but are unable to support nuclease activity (e.g., fluoro- or 2'-O-methoxyethyl- substituted). Chimeric oligonucleotides are not limited to those with modifications on the sugar, but may also include oligonucleosides or oligonucleotides with modified backbones, e.g., with regions of phosphorothioate (P=S) and phosphodiester (P=O) backbone linkages or with regions of MMI and P=S backbone linkages. Other chimeras include "wingmers," also known in the art as "hemimers," that is, oligonucleotides with two distinct regions. In a preferred example of a wingmer, the 5' portion of the oligonucleotide serves as a substrate for RNase H and is preferably composed of 2'-deoxynucleotides, whereas the 3' portion is modified in such a fashion so as

to have greater affinity for the target RNA molecule but is unable to support nuclease activity (e.g., 2'-fluoro- or 2'-O-methoxyethyl- substituted), or vice-versa. In one embodiment, the oligonucleotides of the present invention contain a 2'-O-methoxyethyl (2'-O-$CH_2CH_2OCH_3$) modification on the sugar moiety of at least one nucleotide. This modification has been shown to increase both affinity of the oligonucleotide for its target and nuclease resistance of the oligonucleotide. According to the invention, one, a plurality, or all of the nucleotide subunits of the oligonucleotides of the invention may bear a 2'-O-methoxyethyl (-O-$CH_2CH_2OCH_3$) modification. Oligonucleotides comprising a plurality of nucleotide subunits having a 2'-O-methoxyethyl modification can have such a modification on any of the nucleotide subunits within the oligonucleotide, and may be chimeric oligonucleotides. Aside from or in addition to 2'-O-methoxyethyl modifications, oligonucleotides containing other modifications which enhance antisense efficacy, potency or target affinity are also preferred. Chimeric oligonucleotides comprising one or more such modifications are presently preferred.

The oligonucleotides used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including Applied Biosystems. Any other means for such synthesis may also be employed; the actual synthesis of the oligonucleotides is well within the talents of the routineer. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and 2'-alkoxy or 2'-alkoxyalkoxy derivatives, including 2'-O-methoxyethyl oligonucleotides (Martin, P., Helv. Chim. Acta, 1995, 78, 486-504). It is also well known to use similar techniques and commercially available modified amidites and controlled-pore glass (CPG) products such as

biotin, fluorescein, acridine or psoralen-modified amidites and/or CPG (available from Glen Research, Sterling, VA) to synthesize fluorescently labeled, biotinylated or other conjugated oligonucleotides.

The antisense compounds of the present invention include bioequivalent compounds, including pharmaceutically acceptable salts and prodrugs. This is intended to encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to an animal including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to pharmaceutically acceptable salts of the nucleic acids of the invention and prodrugs of such nucleic acids. "Pharmaceutically acceptable salts" are physiologically and pharmaceutically acceptable salts of the nucleic acids of the invention: *i.e.*, salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto (see, for example, Berge *et al.*, "Pharmaceutical Salts," J. of Pharma Sci., 1977, 66, 1-19).

For oligonucleotides, examples of pharmaceutically acceptable salts include but are not limited to (a) salts formed with cations such as sodium, potassium, ammonium, magnesium, calcium, polyamines such as spermine and spermidine, *etc.*; (b) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; (c) salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, methanesulfonic acid, p-

toluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (d) salts formed from elemental anions such as chlorine, bromine, and iodine.

The oligonucleotides of the invention may additionally or alternatively be prepared to be delivered in a "prodrug" form. The term "prodrug" indicates a therapeutic agent that is prepared in an inactive form that is converted to an active form (*i.e.*, drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or conditions. In particular, prodrug versions of the oligonucleotides of the invention are prepared as SATE [(S-acetyl-2-thioethyl) phosphate] derivatives according to the methods disclosed in WO 93/24510 to Gosselin et al., published December 9, 1993.

For therapeutic or prophylactic treatment, oligonucleotides are administered in accordance with this invention. Oligonucleotide compounds of the invention may be formulated in a pharmaceutical composition, which may include pharmaceutically acceptable carriers, thickeners, diluents, buffers, preservatives, surface active agents, neutral or cationic lipids, lipid complexes, liposomes, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients and the like in addition to the oligonucleotide. Such compositions and formulations are comprehended by the present invention.

Pharmaceutical compositions comprising the oligonucleotides of the present invention may include penetration enhancers in order to enhance the alimentary delivery of the oligonucleotides. Penetration enhancers may be classified as belonging to one of five broad categories, *i.e.*, fatty acids, bile salts, chelating agents, surfactants and non-surfactants (Lee et al., Critical Reviews in Therapeutic Drug Carrier Systems, 1991, 8, 91-192; Muranishi, Critical Reviews in Therapeutic Drug

Carrier Systems, 1990, 7, 1-33). One or more penetration enhancers from one or more of these broad categories may be included.

Various fatty acids and their derivatives which act as penetration enhancers include, for example, oleic acid, lauric acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, recinleate, monoolein (a.k.a. 1-monooleoyl-*rac*-glycerol), dilaurin, caprylic acid, arachidonic acid, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, acylcarnitines, acylcholines, mono- and di-glycerides and physiologically acceptable salts thereof (*i.e.*, oleate, laurate, caprate, myristate, palmitate, stearate, linoleate, etc.) (Lee et al., Critical Reviews in Therapeutic Drug Carrier Systems, 1991, page 92; Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7, 1; El-Hariri et al., J. Pharm. Pharmacol., 1992 *44*, 651-654).

The physiological roles of bile include the facilitation of dispersion and absorption of lipids and fat-soluble vitamins (Brunton, Chapter 38 In: Goodman & Gilman's The Pharmacological Basis of Therapeutics, 9th Ed., Hardman et al., eds., McGraw-Hill, New York, NY, 1996, pages 934-935). Various natural bile salts, and their synthetic derivatives, act as penetration enhancers. Thus, the term "bile salt" includes any of the naturally occurring components of bile as well as any of their synthetic derivatives.

Complex formulations comprising one or more penetration enhancers may be used. For example, bile salts may be used in combination with fatty acids to make complex formulations.

Chelating agents include, but are not limited to, disodium ethylenediaminetetraacetate (EDTA), citric acid, salicylates (e.g., sodium salicylate, 5-methoxysalicylate

and homovanilate), *N*-acyl derivatives of collagen, laureth-9 and *N*-amino acyl derivatives of beta-diketones (enamines)[Lee et al., Critical Reviews in Therapeutic Drug Carrier Systems, 1991, page 92; Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7, 1-33; Buur et al., J. Control Rel., 1990, 14, 43-51). Chelating agents have the added advantage of also serving as DNase inhibitors.

Surfactants include, for example, sodium lauryl sulfate, polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl ether (Lee et al., Critical Reviews in Therapeutic Drug Carrier Systems, 1991, page 92); and perfluorochemical emulsions, such as FC-43 (Takahashi et al., J. Pharm. Phamacol., 1988, 40, 252-257).

Non-surfactants include, for example, unsaturated cyclic ureas, 1-alkyl- and 1-alkenylazacyclo-alkanone derivatives (Lee et al., Critical Reviews in Therapeutic Drug Carrier Systems 1991, page 92); and non-steroidal anti-inflammatory agents such as diclofenac sodium, indomethacin and phenylbutazone (Yamashita et al., J. Pharm. Pharmacol., 1987, 39, 621-626).

As used herein, "carrier compound" refers to a nucleic acid, or analog thereof, which is inert (*i.e.*, does not possess biological activity *per se*) but is recognized as a nucleic acid by *in vivo* processes that reduce the bioavailability of a nucleic acid having biological activity by, for example, degrading the biologically active nucleic acid or promoting its removal from circulation. The coadministration of a nucleic acid and a carrier compound, typically with an excess of the latter substance, can result in a substantial reduction of the amount of nucleic acid recovered in the liver, kidney or other extracirculatory reservoirs, presumably due to competition between the carrier compound and the nucleic acid for a

common receptor. In contrast to a carrier compound, a "pharmaceutically acceptable carrier" (excipient) is a pharmaceutically acceptable solvent, suspending agent or any other pharmacologically inert vehicle for delivering one or more nucleic acids to an animal. The pharmaceutically acceptable carrier may be liquid or solid and is selected with the planned manner of administration in mind so as to provide for the desired bulk, consistency, *etc.*, when combined with a nucleic acid and the other components of a given pharmaceutical composition. Typical pharmaceutically acceptable carriers include, but are not limited to, binding agents (e.g., pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose, etc.); fillers (e.g., lactose and other sugars, microcrystalline cellulose, pectin, gelatin, calcium sulfate, ethyl cellulose, polyacrylates or calcium hydrogen phosphate, etc.); lubricants (e.g., magnesium stearate, talc, silica, colloidal silicon dioxide, stearic acid, metallic stearates, hydrogenated vegetable oils, corn starch, polyethylene glycols, sodium benzoate, sodium acetate, etc.); disintegrates (e.g., starch, sodium starch glycolate, etc.); or wetting agents (e.g., sodium lauryl sulphate, etc.). Sustained release oral delivery systems and/or enteric coatings for orally administered dosage forms are described in U.S. Patents Nos. 4,704,295; 4,556,552; 4,309,406; and 4,309,404.

The compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions, at their art-established usage levels. Thus, for example, the compositions may contain additional compatible pharmaceutically-active materials such as, e.g., antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional materials useful in physically formulating various dosage

forms of the composition of present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the invention.

Regardless of the method by which the oligonucleotides of the invention are introduced into a patient, colloidal dispersion systems may be used as delivery vehicles to enhance the *in vivo* stability of the oligonucleotides and/or to target the oligonucleotides to a particular organ, tissue or cell type. Colloidal dispersion systems include, but are not limited to, macromolecule complexes, nanocapsules, microspheres, beads and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, liposomes and lipid:oligonucleotide complexes of uncharacterized structure. A preferred colloidal dispersion system is a plurality of liposomes. Liposomes are microscopic spheres having an aqueous core surrounded by one or more outer layers made up of lipids arranged in a bilayer configuration (see, generally, Chonn et al., Current Op. Biotech., 1995, 6, 698-708).

The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic, vaginal, rectal, intranasal, epidermal, and transdermal), oral or parenteral. Parenteral administration includes intravenous drip, subcutaneous, intraperitoneal or intramuscular injection, pulmonary administration, e.g., by inhalation or insufflation, or intracranial, e.g., intrathecal or intraventricular, administration. Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be particularly useful for oral administration.

Formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful.

Compositions for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets or tablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable.

Compositions for parenteral administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives. In some cases it may be more effective to treat a patient with an oligonucleotide of the invention in conjunction with other traditional therapeutic modalities in order to increase the efficacy of a treatment regimen. In the context of the invention, the term "treatment regimen" is meant to encompass therapeutic, palliative and prophylactic modalities. For example, a patient may be treated with conventional chemotherapeutic agents, particularly those used for tumor and cancer treatment. Examples of such chemotherapeutic agents include but are not limited to daunorubicin, daunomycin, dactinomycin, doxorubicin, epirubicin, idarubicin, esorubicin, bleomycin, mafosfamide, ifosfamide, cytosine arabinoside, bis-chloroethylnitrosurea, busulfan, mitomycin C, actinomycin D, mithramycin, prednisone, hydroxyprogesterone, testosterone, tamoxifen, dacarbazine, procarbazine, hexamethylmelamine, pentamethylmelamine, mitoxantrone, amsacrine, chlorambucil, methylcyclohexylnitrosurea, nitrogen mustards, melphalan, cyclophosphamide, 6-mercaptopurine, 6-thioguanine, cytarabine (CA), 5-

azacytidine, hydroxyurea, deoxycoformycin, 4-hydroxyperoxycyclophosphoramide, 5-fluorouracil (5-FU), 5-fluorodeoxyuridine (5-FUdR), methotrexate (MTX), colchicine, taxol, vincristine, vinblastine, etoposide, trimetrexate, teniposide, cisplatin and diethylstilbestrol (DES). See, generally, The Merck Manual of Diagnosis and Therapy, 15th Ed., 1987, pp. 1206-1228, Berkow et al., eds., Rahway, N.J. When used with the compounds of the invention, such chemotherapeutic agents may be used individually (e.g., 5-FU and oligonucleotide), sequentially (e.g., 5-FU and oligonucleotide for a period of time followed by MTX and oligonucleotide), or in combination with one or more other such chemotherapeutic agents (e.g., 5-FU, MTX and oligonucleotide, or 5-FU, radiotherapy and oligonucleotide).

The formulation of therapeutic compositions and their subsequent administration is believed to be within the skill of those in the art. Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be estimated based on EC_{50}s found to be effective *in vitro* and in *in vivo* animal models. In general, dosage is from 0.01 μg to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 20 years. Persons of ordinary skill in the art can easily estimate repetition rates for dosing based on measured residence times and concentrations of the drug in bodily fluids or

tissues. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the oligonucleotide is administered in maintenance doses, ranging from 0.01 μg to 100 g per kg of body weight, once or more daily, to once every 20 years.

Thus, in the context of this invention, by "therapeutically effective amount" is meant the amount of the compound which is required to have a therapeutic effect on the treated individual. This amount, which will be apparent to the skilled artisan, will depend upon the age and weight of the individual, the type of disease to be treated, perhaps even the gender of the individual, and other factors which are routinely taken into consideration when designing a drug treatment. A therapeutic effect is assessed in the individual by measuring the effect of the compound on the disease state in the animal. For example, if the disease to be treated is cancer, therapeutic effects are assessed by measuring the rate of growth or the size of the tumor, or by measuring the production of compounds such as cytokines, production of which is an indication of the progress or regression of the tumor.

The following examples illustrate the present invention and are not intended to limit the same.

EXAMPLES

EXAMPLE 1: Synthesis of Oligonucleotides

Unmodified oligodeoxynucleotides are synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidite chemistry with oxidation by iodine. β-cyanoethyldiisopropyl-phosphoramidites are purchased from Applied Biosystems (Foster City, CA). For phosphorothioate oligonucleotides, the standard oxidation bottle was replaced by a 0.2 M solution of ^{3}H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the stepwise thiation of the phosphite linkages. The thiation

cycle wait step was increased to 68 seconds and was followed by the capping step. Cytosines may be 5-methyl cytosines. (5-methyl deoxycytidine phosphoramidites available from Glen Research, Sterling, VA or Amersham Pharmacia Biotech, Piscataway, NJ)

2'-methoxy oligonucleotides are synthesized using 2'-methoxy β-cyanoethyldiisopropyl-phosphoramidites (Chemgenes, Needham, MA) and the standard cycle for unmodified oligonucleotides, except the wait step after pulse delivery of tetrazole and base is increased to 360 seconds. Other 2'-alkoxy oligonucleotides are synthesized by a modification of this method, using appropriate 2'-modified amidites such as those available from Glen Research, Inc., Sterling, VA.

2'-fluoro oligonucleotides are synthesized as described in Kawasaki et al. (J. Med. Chem., 1993, 36, 831-841). Briefly, the protected nucleoside N^6-benzoyl-2'-deoxy-2'-fluoroadenosine is synthesized utilizing commercially available 9-ß-D-arabinofuranosyladenine as starting material and by modifying literature procedures whereby the 2'-α-fluoro atom is introduced by a S_N2-displacement of a 2'-ß-O-trifyl group. Thus N^6-benzoyl-9-ß-D-arabinofuranosyladenine is selectively protected in moderate yield as the 3',5'-ditetrahydropyranyl (THP) intermediate. Deprotection of the THP and N^6-benzoyl groups is accomplished using standard methodologies and standard methods are used to obtain the 5'-dimethoxytrityl- (DMT) and 5'-DMT-3'-phosphoramidite intermediates.

The synthesis of 2'-deoxy-2'-fluoroguanosine is accomplished using tetraisopropyldisiloxanyl (TPDS) protected 9-ß-D-arabinofuranosylguanine as starting material, and conversion to the intermediate diisobutyryl-arabinofuranosylguanosine. Deprotection of the TPDS group is followed by protection of the hydroxyl group with THP to give diisobutyryl di-THP protected arabinofuranosylguanine.

Selective O-deacylation and triflation is followed by treatment of the crude product with fluoride, then deprotection of the THP groups. Standard methodologies are used to obtain the 5'-DMT- and 5'-DMT-3'-phosphoramidites.

Synthesis of 2'-deoxy-2'-fluorouridine is accomplished by the modification of a known procedure in which 2, 2'-anhydro-1-ß-D-arabinofuranosyluracil is treated with 70% hydrogen fluoride-pyridine. Standard procedures are used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

2'-deoxy-2'-fluorocytidine is synthesized via amination of 2'-deoxy-2'-fluorouridine, followed by selective protection to give N^4-benzoyl-2'-deoxy-2'-fluorocytidine. Standard procedures are used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

2'-(2-methoxyethyl)-modified amidites were synthesized according to Martin, P. (Helv. Chim. Acta, 1995, 78, 486-506). For ease of synthesis, the last nucleotide may be a deoxynucleotide. 2'-O-$CH_2CH_2OCH_3$-cytosines may be 5-methyl cytosines.

Synthesis of 5-Methyl cytosine monomers:

<u>2,2'-Anhydro[1-(ß-D-arabinofuranosyl)-5-methyluridine]:</u>

5-Methyluridine (ribosylthymine, commercially available through Yamasa, Choshi, Japan) (72.0 g, 0.279 M), diphenylcarbonate (90.0 g, 0.420 M) and sodium bicarbonate (2.0 g, 0.024 M) were added to DMF (300 mL). The mixture was heated to reflux, with stirring, allowing the evolved carbon dioxide gas to be released in a controlled manner. After 1 hour, the slightly darkened solution was concentrated under reduced pressure. The resulting syrup was poured into diethylether (2.5 L), with stirring. The product formed a gum. The ether was decanted and the residue was dissolved in a minimum amount of methanol (ca. 400 mL). The solution was poured into fresh ether (2.5 L) to yield a stiff gum. The ether was decanted and the gum was dried in a vacuum oven (60°C at 1 mm Hg for 24 h) to

give a solid which was crushed to a light tan powder (57 g, 85% crude yield). The material was used as is for further reactions.

2'-O-Methoxyethyl-5-methyluridine:

2,2'-Anhydro-5-methyluridine (195 g, 0.81 M), tris(2-methoxyethyl)borate (231 g, 0.98 M) and 2-methoxyethanol (1.2 L) were added to a 2 L stainless steel pressure vessel and placed in a pre-heated oil bath at 160°C. After heating for 48 hours at 155-160°C, the vessel was opened and the solution evaporated to dryness and triturated with MeOH (200 mL). The residue was suspended in hot acetone (1 L). The insoluble salts were filtered, washed with acetone (150 mL) and the filtrate evaporated. The residue (280 g) was dissolved in CH_3CN (600 mL) and evaporated. A silica gel column (3 kg) was packed in CH_2Cl_2/acetone/MeOH (20:5:3) containing 0.5% Et_3NH. The residue was dissolved in CH_2Cl_2 (250 mL) and adsorbed onto silica (150 g) prior to loading onto the column. The product was eluted with the packing solvent to give 160 g (63%) of product.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine:

2'-O-Methoxyethyl-5-methyluridine (160 g, 0.506 M) was co-evaporated with pyridine (250 mL) and the dried residue dissolved in pyridine (1.3 L). A first aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the mixture stirred at room temperature for one hour. A second aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the reaction stirred for an additional one hour. Methanol (170 mL) was then added to stop the reaction. HPLC showed the presence of approximately 70% product. The solvent was evaporated and triturated with CH_3CN (200 mL). The residue was dissolved in $CHCl_3$ (1.5 L) and extracted with 2x500 mL of saturated $NaHCO_3$ and 2x500 mL of saturated NaCl. The organic phase was dried over Na_2SO_4, filtered and evaporated. 275 g of residue was obtained. The residue was purified on a 3.5 kg silica gel column, packed and

eluted with EtOAc/Hexane/Acetone (5:5:1) containing 0.5% Et_3NH. The pure fractions were evaporated to give 164 g of product. Approximately 20 g additional was obtained from the impure fractions to give a total yield of 183 g (57%).

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine:

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (106 g, 0.167 M), DMF/pyridine (750 mL of a 3:1 mixture prepared from 562 mL of DMF and 188 mL of pyridine) and acetic anhydride (24.38 mL, 0.258 M) were combined and stirred at room temperature for 24 hours. The reaction was monitored by tlc by first quenching the tlc sample with the addition of MeOH. Upon completion of the reaction, as judged by tlc, MeOH (50 mL) was added and the mixture evaporated at 35°C. The residue was dissolved in $CHCl_3$ (800 mL) and extracted with 2x200 mL of saturated sodium bicarbonate and 2x200 mL of saturated NaCl. The water layers were back extracted with 200 mL of $CHCl_3$. The combined organics were dried with sodium sulfate and evaporated to give 122 g of residue (approx. 90% product). The residue was purified on a 3.5 kg silica gel column and eluted using EtOAc/Hexane(4:1). Pure product fractions were evaporated to yield 96 g (84%).

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine:

A first solution was prepared by dissolving 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (96 g, 0.144 M) in CH_3CN (700 mL) and set aside. Triethylamine (189 mL, 1.44 M) was added to a solution of triazole (90 g, 1.3 M) in CH_3CN (1 L), cooled to -5°C and stirred for 0.5 h using an overhead stirrer. $POCl_3$ was added dropwise, over a 30 minute period, to the stirred solution maintained at 0-10°C, and the resulting mixture stirred for an additional 2 hours. The first solution was added dropwise, over a 45 minute period, to the later

solution. The resulting reaction mixture was stored overnight in a cold room. Salts were filtered from the reaction mixture and the solution was evaporated. The residue was dissolved in EtOAc (1 L) and the insoluble solids were removed by filtration. The filtrate was washed with 1x300 mL of $NaHCO_3$ and 2x300 mL of saturated NaCl, dried over sodium sulfate and evaporated. The residue was triturated with EtOAc to give the title compound.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine:

A solution of 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine (103 g, 0.141 M) in dioxane (500 mL) and NH_4OH (30 mL) was stirred at room temperature for 2 hours. The dioxane solution was evaporated and the residue azeotroped with MeOH (2x200 mL). The residue was dissolved in MeOH (300 mL) and transferred to a 2 liter stainless steel pressure vessel. MeOH (400 mL) saturated with NH_3 gas was added and the vessel heated to 100°C for 2 hours (tlc showed complete conversion). The vessel contents were evaporated to dryness and the residue was dissolved in EtOAc (500 mL) and washed once with saturated NaCl (200 mL). The organics were dried over sodium sulfate and the solvent was evaporated to give 85 g (95%) of the title compound.

N^4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-cytidine:

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyl-cytidine (85 g, 0.134 M) was dissolved in DMF (800 mL) and benzoic anhydride (37.2 g, 0.165 M) was added with stirring. After stirring for 3 hours, tlc showed the reaction to be approximately 95% complete. The solvent was evaporated and the residue azeotroped with MeOH (200 mL). The residue was dissolved in $CHCl_3$ (700 mL) and extracted with saturated $NaHCO_3$ (2x300 mL) and saturated NaCl (2x300 mL), dried over $MgSO_4$ and evaporated to give a residue (96 g). The residue was chromatographed on a 1.5 kg silica

column using EtOAc/Hexane (1:1) containing 0.5% Et_3NH as the eluting solvent. The pure product fractions were evaporated to give 90 g (90%) of the title compound.

<u>N^4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine-3'-amidite:</u>

N^4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (74 g, 0.10 M) was dissolved in CH_2Cl_2 (1 L). Tetrazole diisopropylamine (7.1 g) and 2-cyanoethoxy-tetra(isopropyl)phosphite (40.5 mL, 0.123 M) were added with stirring, under a nitrogen atmosphere. The resulting mixture was stirred for 20 hours at room temperature (tlc showed the reaction to be 95% complete). The reaction mixture was extracted with saturated $NaHCO_3$ (1x300 mL) and saturated NaCl (3x300 mL). The aqueous washes were back-extracted with CH_2Cl_2 (300 mL), and the extracts were combined, dried over $MgSO_4$ and concentrated. The residue obtained was chromatographed on a 1.5 kg silica column using EtOAc\Hexane (3:1) as the eluting solvent. The pure fractions were combined to give 90.6 g (87%) of the title compound.

5-methyl-2'-deoxycytidine (5-me-C) containing oligonucleotides were synthesized according to published methods (Sanghvi et al., Nucl. Acids Res., 1993, 21, 3197-3203) using commercially available phosphoramidites (Glen Research, Sterling VA or ChemGenes, Needham MA).

2'-O-(dimethylaminooxyethyl) nucleoside amidites

2'-(Dimethylaminooxyethoxy) nucleoside amidites [also known in the art as 2'-O-(dimethylaminooxyethyl) nucleoside amidites] are prepared as described in the following paragraphs. Adenosine, cytidine and guanosine nucleoside amidites are prepared similarly to the thymidine (5-methyluridine) except the exocyclic amines are protected with a benzoyl moiety in the case of adenosine and cytidine and with isobutyryl in the case of guanosine.

5'-O-tert-Butyldiphenylsilyl-O^2-2'-anhydro-5-methyluridine

O^2-2'-anhydro-5-methyluridine (Pro. Bio. Sint., Varese, Italy, 100.0g, 0.416 mmol), dimethylaminopyridine (0.66g, 0.013eq, 0.0054mmol) were dissolved in dry pyridine (500 ml) at ambient temperature under an argon atmosphere and with mechanical stirring. *tert*-Butyldiphenylchlorosilane (125.8g, 119.0mL, 1.1eq, 0.458mmol) was added in one portion. The reaction was stirred for 16 h at ambient temperature. TLC (Rf 0.22, ethyl acetate) indicated a complete reaction. The solution was concentrated under reduced pressure to a thick oil. This was partitioned between dichloromethane (1 L) and saturated sodium bicarbonate (2x1 L) and brine (1 L). The organic layer was dried over sodium sulfate and concentrated under reduced pressure to a thick oil. The oil was dissolved in a 1:1 mixture of ethyl acetate and ethyl ether (600mL) and the solution was cooled to -10°C. The resulting crystalline product was collected by filtration, washed with ethyl ether (3x200 mL) and dried (40°C, 1mm Hg, 24 h) to 149g (74.8%) of white solid. TLC and NMR were consistent with pure product.

5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine

In a 2 L stainless steel, unstirred pressure reactor was added borane in tetrahydrofuran (1.0 M, 2.0 eq, 622 mL). In the fume hood and with manual stirring, ethylene glycol (350 mL, excess) was added cautiously at first until the evolution of hydrogen gas subsided. 5'-O-tert-Butyldiphenylsilyl-O^2-2'-anhydro-5-methyluridine (149 g, 0.311 mol) and sodium bicarbonate (0.074 g, 0.003 eq) were added with manual stirring. The reactor was sealed and heated in an oil bath until an internal temperature of 160 °C was reached and then maintained for 16 h (pressure < 100 psig). The reaction vessel was cooled to ambient and opened. TLC (Rf 0.67 for desired product and Rf 0.82 for

ara-T side product, ethyl acetate) indicated about 70% conversion to the product. In order to avoid additional side product formation, the reaction was stopped, concentrated under reduced pressure (10 to 1mm Hg) in a warm water bath (40-100°C) with the more extreme conditions used to remove the ethylene glycol. [Alternatively, once the low boiling solvent is gone, the remaining solution can be partitioned between ethyl acetate and water. The product will be in the organic phase.] The residue was purified by column chromatography (2kg silica gel, ethyl acetate-hexanes gradient 1:1 to 4:1). The appropriate fractions were combined, stripped and dried to product as a white crisp foam (84g, 50%), contaminated starting material (17.4g) and pure reusable starting material 20g. The yield based on starting material less pure recovered starting material was 58%. TLC and NMR were consistent with 99% pure product.

<u>2'-O-([2-phthalimidoxy)ethyl]-5'-*t*-butyldiphenylsilyl-5-methyluridine</u>

5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine (20g, 36.98mmol) was mixed with triphenylphosphine (11.63g, 44.36mmol) and N-hydroxyphthalimide (7.24g, 44.36mmol). It was then dried over P_2O_5 under high vacuum for two days at 40°C. The reaction mixture was flushed with argon and dry THF (369.8mL, Aldrich, sure seal bottle) was added to get a clear solution. Diethyl-azodicarboxylate (6.98mL, 44.36mmol) was added dropwise to the reaction mixture. The rate of addition is maintained such that resulting deep red coloration is just discharged before adding the next drop. After the addition was complete, the reaction was stirred for 4 hrs. By that time TLC showed the completion of the reaction (ethylacetate:hexane, 60:40). The solvent was evaporated in vacuum. Residue obtained was placed on a flash column and eluted with ethyl acetate:hexane (60:40),

to get 2'-O-([2-phthalimidoxy)ethyl]-5'-*t*-butyldiphenylsilyl-5-methyluridine as white foam (21.819, 86%).

<u>5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine</u>

2'-O-([2-phthalimidoxy)ethyl]-5'-*t*-butyldiphenylsilyl-5-methyluridine (3.1g, 4.5mmol) was dissolved in dry CH_2Cl_2 (4.5mL) and methylhydrazine (300mL, 4.64mmol) was added dropwise at -10°C to 0°C. After 1 hr the mixture was filtered, the filtrate was washed with ice cold CH_2Cl_2 and the combined organic phase was washed with water, brine and dried over anhydrous Na_2SO_4. The solution was concentrated to get 2'-*O*-(aminooxyethyl) thymidine, which was then dissolved in MeOH (67.5mL). To this formaldehyde (20% aqueous solution, w/w, 1.1eg.) was added and the mixture for 1 hr. Solvent was removed under vacuum; residue chromatographed to get 5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy) ethyl]-5-methyluridine as white foam (1.95, 78%).

<u>5'-O-*tert*-Butyldiphenylsilyl-2'-O-[N,N-dimethylaminooxyethyl]-5-methyluridine</u>

5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine (1.77g, 3.12mmol) was dissolved in a solution of 1M pyridinium p-toluenesulfonate (PPTS) in dry MeOH (30.6mL). Sodium cyanoborohydride (0.39g, 6.13mmol) was added to this solution at 10°C under inert atmosphere. The reaction mixture was stirred for 10 minutes at 10°C. After that the reaction vessel was removed from the ice bath and stirred at room temperature for 2 hr, the reaction monitored by TLC (5% MeOH in CH_2Cl_2). Aqueous $NaHCO_3$ solution (5%, 10mL) was added and extracted with ethyl acetate (2x20mL). Ethyl acetate phase was dried over anhydrous Na_2SO_4, evaporated to dryness. Residue was dissolved in a solution of 1M PPTS in MeOH (30.6mL). Formaldehyde (20% w/w, 30mL, 3.37mmol) was

added and the reaction mixture was stirred at room temperature for 10 minutes. Reaction mixture cooled to 10°C in an ice bath, sodium cyanoborohydride (0.39g, 6.13mmol) was added and reaction mixture stirred at 10°C for 10 minutes. After 10 minutes, the reaction mixture was removed from the ice bath and stirred at room temperature for 2 hrs. To the reaction mixture 5% $NaHCO_3$ (25mL) solution was added and extracted with ethyl acetate (2x25mL). Ethyl acetate layer was dried over anhydrous Na_2SO_4 and evaporated to dryness . The residue obtained was purified by flash column chromatography and eluted with 5% MeOH in CH_2Cl_2 to get 5'-O-*tert*-butyldiphenylsilyl-2'-O-[N,N-dimethylaminooxyethyl]-5-methyluridine as a white foam (14.6g, 80%).

<u>2'-O-(dimethylaminooxyethyl)-5-methyluridine</u>

Triethylamine trihydrofluoride (3.91mL, 24.0mmol) was dissolved in dry THF and triethylamine (1.67mL, 12mmol, dry, kept over KOH). This mixture of triethylamine-2HF was then added to 5'-O-*tert*-butyldiphenylsilyl-2'-O-[N,N-dimethylaminooxyethyl]-5-methyluridine (1.40g, 2.4mmol) and stirred at room temperature for 24 hrs. Reaction was monitored by TLC (5% MeOH in CH_2Cl_2). Solvent was removed under vacuum and the residue placed on a flash column and eluted with 10% MeOH in CH_2Cl_2 to get 2'-O-(dimethylaminooxyethyl)-5-methyluridine (766mg, 92.5%).

<u>5'-O-DMT-2'-O-(dimethylaminooxyethyl)-5-methyluridine</u>

2'-O-(dimethylaminooxyethyl)-5-methyluridine (750mg, 2.17mmol) was dried over P_2O_5 under high vacuum overnight at 40°C. It was then co-evaporated with anhydrous pyridine (20mL). The residue obtained was dissolved in pyridine (11mL) under argon atmosphere. 4-dimethylaminopyridine (26.5mg, 2.60mmol), 4,4'-dimethoxytrityl chloride (880mg, 2.60mmol) was added to the mixture and the reaction mixture was stirred at room temperature until all of the starting material disappeared. Pyridine was removed under vacuum

and the residue chromatographed and eluted with 10% MeOH in CH_2Cl_2 (containing a few drops of pyridine) to get 5'-O-DMT-2'-O-(dimethylamino-oxyethyl)-5-methyluridine (1.13g, 80%).

5'-O-DMT-2'-O-(2-N,N-dimethylaminooxyethyl)-5-methyluridine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite]

5'-O-DMT-2'-O-(dimethylaminooxyethyl)-5-methyluridine (1.08g, 1.67mmol) was co-evaporated with toluene (20mL). To the residue N,N-diisopropylamine tetrazonide (0.29g, 1.67mmol) was added and dried over P_2O_5 under high vacuum overnight at 40°C. Then the reaction mixture was dissolved in anhydrous acetonitrile (8.4mL) and 2-cyanoethyl-N,N,N^1,N^1-tetraisopropylphosphoramidite (2.12mL, 6.08mmol) was added. The reaction mixture was stirred at ambient temperature for 4 hrs under inert atmosphere. The progress of the reaction was monitored by TLC (hexane:ethyl acetate 1:1). The solvent was evaporated, then the residue was dissolved in ethyl acetate (70mL) and washed with 5% aqueous $NaHCO_3$ (40mL). Ethyl acetate layer was dried over anhydrous Na_2SO_4 and concentrated. Residue obtained was chromatographed (ethyl acetate as eluent) to get 5'-O-DMT-2'-O-(2-N,N-dimethylaminooxyethyl)-5-methyluridine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite] as a foam (1.04g, 74.9%).

Oligonucleotides having methylene(methylimino) (MMI) backbones are synthesized according to U.S. Patent 5,378,825, which is coassigned to the assignee of the present invention and is incorporated herein in its entirety. For ease of synthesis, various nucleoside dimers containing MMI linkages were synthesized and incorporated into oligonucleotides. Other nitrogen-containing backbones are synthesized according to WO 92/20823 which is also coassigned to the assignee of the present invention and incorporated herein in its entirety.

Oligonucleotides having amide backbones are synthesized according to De Mesmaeker et al. (Acc. Chem. Res., 1995, 28, 366-374). The amide moiety is readily accessible by simple and well-known synthetic methods and is compatible with the conditions required for solid phase synthesis of oligonucleotides.

Oligonucleotides with morpholino backbones are synthesized according to U.S. Patent 5,034,506 (Summerton and Weller).

Peptide-nucleic acid (PNA) oligomers are synthesized according to P.E. Nielsen et al. (Science, 1991, 254, 1497-1500).

After cleavage from the controlled pore glass column (Applied Biosystems) and deblocking in concentrated ammonium hydroxide at 55°C for 18 hours, the oligonucleotides are purified by precipitation twice out of 0.5 M NaCl with 2.5 volumes ethanol. Synthesized oligonucleotides were analyzed by polyacrylamide gel electrophoresis on denaturing gels and judged to be at least 85% full length material. The relative amounts of phosphorothioate and phosphodiester linkages obtained in synthesis were periodically checked by ^{31}P nuclear magnetic resonance spectroscopy, and for some studies oligonucleotides were purified by HPLC, as described by Chiang et al. (J. Biol. Chem., 1991, 266, 18162). Results obtained with HPLC-purified material were similar to those obtained with non-HPLC purified material.

Example 2: Oligonucleotide Sequences and Cell Culture

Antisense oligonucleotides were designed to E-selectin, Ha-*ras*, *c-raf*, JNK1, and JNK2. Additional sequences were designed as scrambled controls. The sequence of the oligonucleotides used are given in Table 1. All of these oligonucleotides except ISIS 11928 (SEQ ID NO. 1) contain 2'-O-methoxyethyl/phosphodiester residues flanking a 2'-deoxynucleotide/phosphorothioate region.

ISIS 11928 (SEQ ID NO. 1) is a fully phosphorothioated oligonucleotide with all 2'-methoxyethoxy nucleotides, except for a 3' terminal 2'-deoxy nucleotide. The target sequence for E-selectin was obtained from the Genbank endothelial leukocyte adhesion molecule I exon 1 sequence, HUMELAM1 (Accession number M61895; SEQ ID NO. 8). The target sequence for Ha-*ras* was obtained from the Genbank Ha-*ras* sequence, HSRAS1 (Accession number V00574; SEQ ID NO. 10). The target sequence for *c-raf* was obtained from the Genbank *c-raf* sequence, HSRAFR (Accession number X03484; SEQ ID NO. 12). The target sequence for JNK1 was obtained from the Genbank JNK1 sequence, HUMJNK1 (Accession number L26318; SEQ ID NO. 14). The target sequence for JNK2 was obtained from the Genbank JNK2 sequence, HUMJNK2 (Accession number L31951; SEQ ID NO. 16).

TABLE 1

Nucleotide Sequences of Mixed Backbone Chimeric (deoxy gapped) 2'-O-methoxyethyl Oligonucleotides

ISIS NO.	NUCLEOTIDE SEQUENCE (5' -> 3')[1]	SEQ ID NO:	TARGET GENE
11928	GsAsAsGsTsCsAsGsCsCsAsAsGsAsAsCsAsGsCsT	1	E-selectin
12854	ToCoCoCoGoCsCsTsGsTsGsAsCsAsToGoCoAoToT	2	*c-raf*
15727	AoToGoCoAoTsTsCsTsGsCsCsCsCsCoAoAoGoGoA	3	12854 control
15168	ToCoCsGsTsCsAsTsCsGsCsTsCoCoToCoAoGoGoG	4	Ha-*ras*
17552	ToCoAsGsTsAsAsTsAsGsCsCsCoCoAoCoAoToGoG	5	15168 Control
15347	CoToCoToCoTsGsTsAsGsGsCsCsCsGsCoToToGoG	6	JNK1
15354	GoToCoCoGoGsGsCsCsAsGsGsCsCsAsAoAoGoToC	7	JNK2

[1] Underlined residues are 2'-methoxyethoxy residues (others are 2'-deoxy-). All 2'-methoxyethoxy cytidines are 5-methyl-cytidines; "s" linkages are phosphorothioate linkages, "o" linkages are phosphodiester linkages.

Human dermal microvascular cells (HMVEC-d; Clonetics, San Diego, CA) were cultivated in endothelial basal media (EBM, Clonetics) supplemented with 10% fetal bovine serum (HyClone, Logan, UT). Cells were grown in 100 mm petri dishes until 70-80% confluent, then washed with PBS and OPTI-MEM (Life Technologies, Inc., Gaithersburg, MD). The cells were then incubated in the presence of OPTI-MEM and 3 mg/ml LIPOFECTIN (a 1:1 (w/w) liposome formulation of the cationic lipid N-[1-(2,3-dioleyloxy)propyl]-n,n,n-trimethylammonium chloride (DOTMA), and dioleoyl phosphotidylethanolamine (DOPE) in membrane filtered water (Life Technologies, Gaithersburg, MD), per 100 nM of oligonucleotide followed by addition of oligonucleotide at the appropriate concentrations.

For determination of mRNA levels by Northern blot, total RNA was prepared from cells by the guanidinium isothiocyanate procedure or by the Qiagen RNAEASY method (Qiagen, Santa Clarita, CA) at the indicated times after initiation of oligonucleotide treatment. Northern blot analysis was performed as described in Current Protocols in Molecular Biology (Ausubel, F.M., et al., (eds), 1987, Greene Publishing Assoc. and Wiley Interscience, New York). A glyceraldehyde 3-phosphate dehydrogenase (G3PDH) probe was purchased from Clontech (Palo Alto, CA, Catalog Number 9805-1). RNA was quantified and normalized to G3PDH mRNA levels using a Molecular Dynamic PHOSPHORIMAGER (Sunnyvale, CA).

For determination of protein levels by Western blot, cellular extracts were prepared using 300 ml of RIPA extraction buffer per 100-mm dish. The protein concentration was quantified by Bradford assay using the BioRad kit (BioRad, Hercules, CA). Equal amounts of protein were loaded on 10% or 12% SDS-PAGE mini-gel (Novex, San Diego, CA). Once transferred to PVDF membranes (Millipore, Bedford, MA), the membranes were then treated

for a minimum of 2 hours with specific primary antibody followed by incubation with secondary antibody conjugated to HRP. The results were visualized by Enhanced Chemiluminescent (ECL) detection (New England BioLab, Beverly, MA). In some experiments, the blots were stripped in stripping buffer (2% SDS, 12.5 mM Tris, pH 6.8) for 30 minutes at 50°C. After extensive washing, the blots were blocked and blotted with different primary antibody.

Example 3: Inhibition of *c-raf* and Ha-*ras* Expression by Antisense Oligonucleotides

ISIS 12854 (SEQ ID NO. 2) is a 2'-O-methoxyethyl mixed backbone chimeric antisense oligonucleotide designed to hybridize with 3'-untranslated sequences contained within human *c-raf* mRNA. To determine whether this antisense oligonucleotide is effective as a *c-raf* inhibitor in endothelial cells, human dermal microvascular cells (HMVEC) were treated with ISIS 12854 (SEQ ID NO. 2) as described in Example 2. *C-raf* mRNA and protein expression was examined by northern and Western blot analysis. The *c-raf* cDNA probe was generated from the plasmid, p627 (American Type Culture Collection, Manassas, VA; catalog #41050), following the supplied instructions. The *c-raf* antibody was obtained from Transduction Laboratories, Inc. (Lexington, KY). Northern blot results are shown in Table 2. Western blot results are shown in Figure 1.

TABLE 2

Dose Response of ISIS 12854 on *c-raf* mRNA Levels in HMVEC Cells

ISIS #	SEQ ID NO:	ASO Gene Target	Dose	% mRNA Expression	% mRNA Inhibition
LIPOFECTIN	---	---	---	100%	---
15727	3	control	100 nM	96%	4%

"	"	"	200 nM	135%	---
12854	2	*c-raf*	0.5 nM	67%	33%
"	"	"	2.5 nM	31%	69%
"	"	"	10 nM	15%	85%
"	"	"	50 nM	10%	90%
"	"	"	100 nM	13%	87%
"	"	"	200 nM	3%	97%

Treatment of HMVEC with ISIS 12854 (SEQ ID NO. 2) resulted in dramatically reduced *c-raf* mRNA levels. Reduction of *c-raf* mRNA levels was dose-dependent in the range of 0.5 to 200 nM. The IC_{50} for *c-raf* mRNA reduction was approximately 2.5 nM. The control oligonucleotide, ISIS 15727 (SEQ ID NO. 3) did not exhibit any effect on *c-raf* mRNA.

Reduction of *c-raf* protein levels also occurred following oligonucleotide treatment. Reduction in protein expression was first detectable 24 hours after treatment and maximal reduction of *c-raf* protein levels was achieved 48 hours after treatment with 150 nM oligonucleotide. Reduction of *c-raf* protein levels persisted up to 72 hour following initial treatment. Inhibition of *c-raf* expression by ISIS 12854 (SEQ ID NO. 2) was specific since A-*raf* protein expression was largely unaffected when the same blot was stripped and blotted with antibody against A-*raf* (Transduction Laboratories, Inc., Lexington, KY).

ISIS 15168 (SEQ ID NO. 4) is a 2'-O-methoxyethyl mixed backbone chimeric antisense oligonucleotide designed to hybridize with sequences contained within the human Ha-*ras* mRNA. To determine the effect of ISIS 15168 (SEQ ID NO. 4) on Ha-*ras* expression in endothelial cells, HMVEC were treated, as described in Example 2, with oligonucleotide and northern blotting was used to measure Ha-*ras* mRNA levels. A Ha-*ras* probe was generated from the plasmid,

pbc-N1 (American Type Culture Collection, Manassas, VA; catalog #41001), following the supplied instructions. Results are shown in Table 3.

TABLE 3

Dose Response of Ha-*ras* Antisense Oligodeoxynucleotides on Ha-*ras* mRNA Levels in HMVEC Cells

ISIS #	SEQ ID NO:	ASO Gene Target	Dose	% mRNA Expression	% mRNA Inhibition
LIPOFECTIN™	---	---	---	100%	---
15168	4	Ha-*ras*	1 nM	61%	39%
"	"	"	5 nM	44%	56%
"	"	"	25 nM	19%	81%
"	"	"	50 nM	10%	90%
"	"	"	100 nM	9%	91%
17552	5	control	1 nM	103%	---
"	"	"	5 nM	115%	---
"	"	"	25 nM	96%	4%
"	"	"	50 nM	109%	---
"	"	"	100 nM	105%	---

Reduction of Ha-*ras* mRNA levels in HMVEC was observed following oligonucleotide treatment and was found to be sequence-specific, and dose-dependent (IC_{50} < 5 nM).

To examine the effect of ISIS 15168 (SEQ ID NO. 4) on Ha-*ras* protein levels, total *ras* protein was immunoprecipitated using a pan-*ras* monoclonal antibody (Oncogene Science, Cambridge, MA). The precipitated proteins were analyzed by SDS-PAGE, and Ha-*ras* protein levels were determined by western blot using a monoclonal antibody specific for Ha-*ras* (Oncogene Science, Cambridge, MA). As shown in Figure 2, dose-dependent reduction of Ha-*ras* protein expression was observed 48 hours following treatment with ISIS 15168 (SEQ ID NO. 4). This reduction

persisted up to 72 hours following initial treatment. The kinetics of Ha-*ras* reduction was slower than that of *c-raf*. The control oligonucleotide , ISIS 17552 (SEQ ID NO. 5), had no effect on Ha-*ras* protein level. The same blot was stripped and blotted a second time with a Ki-*ras*-specific antibody (Oncogene Science, Cambridge, MA). No effect on Ki-*ras* protein levels was observed in cells treated with either ISIS 15168 (SEQ ID NO. 4) or the control oligonucleotide, ISIS 17552 (SEQ ID NO. 5).

Example 4: Effect of inhibiting *c-raf* and Ha-*ras* Gene Expression on the Induction of E-selectin

The effect of *c-raf* and Ha-*ras* antisense oligonucleotide treatment on the induction of E-selectin by TNF-α was examined. HMVEC cells were treated with either the *c-raf* or Ha-*ras* antisense compound under dose-response conditions or over time at a single dose level followed by stimulation of E-selectin expression by TNF-α for 5 hours. The cell surface expression of E-selectin was determined by flow cytometry analysis. Following oligonucleotide treatment, cells were detached from the plates and analyzed for surface expression of cell adhesion molecules using a Becton Dickinson (San Jose, CA) FACScan. TNF-α and FITC conjugated antibody for E-selectin were obtained from R & D Systems (Minneapolis, MN). PE-conjugated antibody for ICAM-1 was obtained from Pharmingen (San Diego, CA). VCAM-1 antibody was obtained from Santa Cruz Biotechnology, Santa Cruz, CA. Cell surface expression was calculated using the mean value of fluorescence intensity using 3,000-5,000 cells stained with the appropriate antibody for each sample and time point. Results are expressed as percentage of control (cell surface expression induced by TNF-α in cells that were not treated with oligonucleotides) based upon mean fluorescence intensity. Results are shown in Tables 4 and 5. Basal expression of E-selectin and VCAM-1

is undetectable in the absence of TNF-α whereas a low level of basal expression is detectable for ICAM-1.

ABLE 4

Dose Response of the effect of *c-raf* and Ha-*ras* Antisense Oligonucleotides in Induction of E-selectin

ISIS #	SEQ ID NO:	ASO Gene Target	Dose	% Cell Surface Expression	% Cell Surface Inhibition
LIPOFECTIN™	---	---	---	100%	---
12854	2	*c-raf*	25 nM	37%	63%
"	"	"	75 nM	26%	74%
"	"	"	150 nM	23%	77%
15727	3	control	25 nM	119%	---
"	"	"	75 nM	94%	6%
"	"	"	150 nM	106%	---
15168	4	Ha-*ras*	25 nM	72%	28%
"	"	"	75 nM	47%	53%
"	"	"	150 nM	41%	59%
17552	5	control	25 nM	134%	---
"	"	"	75 nM	116%	---
"	"	"	150 nM	116%	---

Dose-dependent inhibition of E-selectin cell surface expression was observed in cells treated with both antisense compounds, ISIS 12854 (SEQ ID NO. 2), targeted to *c-raf* and ISIS 15168 (SEQ ID NO. 4) targeted to Ha-*ras*. Maximal inhibition of E-selectin induction was approximately 80% for ISIS 12854 (SEQ ID NO. 2) and 60% for ISIS 15168 (SEQ ID NO. 4). Control oligonucleotides (SEQ ID NO. 3 and SEQ ID NO. 5) exhibited little to no effect on E-selectin induction. These results indicate that reduction of *c-raf* or Ha-*ras* protein levels blocked the induction of E-selectin by TNF-α in HMVEC.

The effects of *c-raf*, Ha-*ras*, and a E-selectin antisense oligonucleotide on TNF-α-induced E-selectin cell

surface expression under kinetic conditions were examined. If the inhibition of E-selectin induction by the *c-raf* and Ha-*ras* antisense compounds was dependent on the reduction of *c-raf* and Ha-*ras* protein levels, the kinetics of inhibition of E-selectin induction by the antisense compounds should correlate with suppression of *c-raf* and Ha-*ras* protein levels (which is dependent on the half-lives of the proteins in cells). Inhibition of E-selectin induction using the E-selectin antisense oligonucleotide should occur much more quickly since this inhibitor blocks E-selectin induction directly. To test this, cells were treated, separately, with antisense oligonucleotides to E-selectin, ICAM-1 and VCAM-1 and allowed to recover prior to TNF-α treatment. TNF-α was added at different time points between 12 and 72 hours following oligonucleotide treatment. E-selectin cell surface expression was measured by flow cytometry analysis after 5 hours of TNF-α treatment. Results are shown in Table 5.

TABLE 5

Time Course of the Effect of *c-raf* and Ha-*ras* Antisense Oligonucleotides in Induction of E-selectin

ISIS #	SEQ ID NO:	ASO Gene Target	Time (hours)	% Cell Surface Expression	% Cell Surface Inhibition
LIPOFECTIN	---	---	---	100%	---
11928	1	E-selectin	12h/20h	22%	78%
"	"	"	48h	29%	71%
"	"	"	72h	32%	68%
12854	2	*c-raf*	12h/20h	58%	42%
"	"	"	48h	23%	77%
"	"	"	72h	24%	76%
15727	3	control	12h/20h	93%	7%

"	"	"	48h	106%	---
"	"	"	72h	81%	19%
15168	4	Ha-*ras*	12h/20h	87%	13%
"	"	"	48h	42%	58%
"	"	"	72h	42%	58%
17552	5	control	12h/20h	113%	---
"	"	"	48h	116%	---
"	"	"	72h	111%	---

Concentrations as low as 20 nM of the E-selectin oligonucleotide, ISIS 11928 (SEQ ID NO. 1) were found to block 80% of E-selectin cell surface expression 12 hours following treatment. In contrast, maximal inhibition of E-selectin induction by *c-raf* and Ha-*ras* antisense compounds, SEQ ID NO. 2 and SEQ ID NO. 4, respectively, was observed 48 hours following antisense treatment. Some inhibition of E-selectin induction was observed at 12 hours following treatment, but this inhibition was clearly not maximal. These results strongly suggest that inhibition of E-selectin induction by TNF-α in HMVEC following treatment with *c-raf* and Ha-*ras* antisense oligonucleotides is a consequence of reduced *c-raf* and Ha-*ras* protein levels.

Example 5: Effect of *c-raf* and Ha-*ras* Antisense Oligonucleotides on the Induction of other Cell Adhesion Molecules

TNF-α induction of ICAM-1 and VCAM-1 in cells treated with *c-raf* and Ha-*ras* antisense oligonucleotides was also examined to further define the roles of *c-raf* and Ha-*ras* in cytokine signaling. Oligonucleotides were tested, using flow cytometry analysis, as described in Example 4. Results are shown in Table 6 (ICAM-1) and Table 7 (VCAM-1).

TABLE 6

Dose response of the effect of *c-raf* and Ha-*ras* antisense oligonucleotides in induction of ICAM-1

ISIS #	SEQ ID NO:	ASO Gene Target	Dose (conc.)	% Cell Surface Expression	% Cell Surface Inhibition
LIPOFECTIN™	---	---	---	100%	---
12854	2	*c-raf*	20 nM	80%	20%
"	"	"	50 nM	62%	38%
"	"	"	100 nM	57%	43%
15727	3	control	20 nM	97%	3%
"	"	"	50 nM	89%	11%
"	"	"	100 nM	87%	13%
15168	4	Ha-*ras*	20 nM	81%	19%
"	"	"	50 nM	62%	38%
"	"	"	100 nM	54%	46%
17552	5	control	20 nM	102%	---
"	"	"	50 nM	100%	---
"	"	"	100 nM	98%	2%

Induction of ICAM-1 by TNF-α was also blocked by *c-raf* (ISIS 12854, SEQ ID NO. 2) and Ha-*ras* (ISIS 15168, SEQ ID NO. 4) antisense oligonucleotides, with maximal inhibition greater than 40%.

Treatment of cells with *c-raf* and Ha-*ras* antisense oligonucleotides inhibited VCAM-1 expression in a dose-dependent fashion as well. Results are shown in Table 7.

TABLE 7

Dose response of the effect of *c-raf* and Ha-*ras* antisense oligonucleotides in induction of VCAM-1

ISIS #	SEQ ID NO:	ASO Gene Target	Dose (conc.)	% Cell Surface Expression	% Cell Surface Inhibition
LIPOFECTIN™	---	---	---	100%	---
12854	2	*c-raf*	20 nM	46%	54%
"	"	"	50 nM	37%	63%
"	"	"	100 nM	28%	72%
15727	3	control	20 nM	69%	31%
"	"	"	50 nM	81%	19%
"	"	"	100 nM	74%	26%
15168	4	Ha-*ras*	20 nM	70%	30%
"	"	"	50 nM	51%	49%
"	"	"	100 nM	44%	56%
17552	5	control	20 nM	111%	---
"	"	"	50 nM	97%	3%
"	"	"	100 nM	85%	15%

Induction of VCAM-1 by TNF-α was also blocked by *c-raf* (ISIS 12854, SEQ ID NO. 2) and Ha-*ras* (ISIS 15168, SEQ ID NO. 4) antisense oligonucleotides. Maximum inhibition for ISIS 12854 (SEQ ID NO. 2) was greater than 70%, while maximum inhibition for ISIS 15168 (SEQ ID NO. 4) was greater than 50%.

Example 6: Effect of *c-raf* and Ha-*ras* Antisense Oligonucleotides on Cell Adhesion Molecule mRNA levels

Northern blot analysis was carried out to examine whether *c-raf* and Ha-*ras* antisense oligonucleotides inhibit the induction of cell adhesion molecules at the transcriptional level. Cells were treated with *c-raf* (ISIS

12854, SEQ ID NO. 2) or Ha-*ras* (ISIS 15168, SEQ ID NO. 4) antisense oligonucleotides and allowed to recover for 48 hrs. TNF-α was added two to three hours prior to RNA analysis and Northern blotting was performed with probes specific for E-selectin, ICAM-1, and VCAM-1. The probe for E-selectin was obtained by PCR amplification using primers directed to HUMELAM1A (Genbank Accession No. M24736; SEQ ID NO. 18).

The probe for ELAM-1 was obtained by PCR amplification using the following primers:

FORWARD 5'-TTGAAGTCATGATTGCTTCACAGTT-3' SEQ ID NO. 20
REVERSE 5'-TTCTGATTCTTTTGAACTTAAAGGAT-3' SEQ ID NO. 21

The probe for ICAM-1 was obtained by PCR amplification using the following primers:

FORWARD 5'-CGCGGATCCGCGTACTCAGAGTT-3' SEQ ID NO. 22
REVERSE 5'-CGGAATTCCGTTCAGGGAGGCGT-3' SEQ ID NO. 23

The probe for VCAM-1 was obtained by PCR amplification using the following primers:

FORWARD 5'-CTTAAAATGCCTGGGAAGATGGTCGT-3' SEQ ID NO. 24
REVERSE 5'-ATCAAGCATTAGCTACACTTTTGATT-3' SEQ ID NO. 25

Results are shown in Table 8.

TABLE 8

Effect of *c-raf* and Ha-*ras* antisense oligonucleotides on induction of E-selectin, VCAM-1, and ICAM-1

ISIS #	SEQ ID NO:	ASO Gene Target	Cell adhesion molecule	% mRNA Expression	% mRNA Inhibition
LIPOFECTIN™	---	---	---	100%	---
12854	2	*c-raf*	E-selectin	22%	78%
"	"	"	ICAM-1	78%	22%
"	"	"	VCAM-1	35%	65%
15727	3	control	E-selectin	103%	---

"	"	"	ICAM-1	107%	---
"	"	"	VCAM-1	113%	---
15168	4	Ha-*ras*	E-selectin	13%	87%
"	"	"	ICAM-1	73%	27%
"	"	"	VCAM-1	29%	71%
17552	5	control	E-selectin	92%	8%
"	"	"	ICAM-1	101%	---
"	"	"	VCAM-1	111%	---

Induction of mRNA expression of the three cell adhesion molecules was attenuated by both antisense oligonucleotides, whereas treatment with control oligonucleotides exhibited little to no effect. The level of inhibition for each cell adhesion molecule at the mRNA level was consistent with the effects of *c-raf* and Ha-*ras* antisense treatment on the cell surface expression of cell adhesion molecules.

Example 7: Effect of *c-raf* Antisense Oligonucleotides on MAP Kinase Activities

To examine the effect of the *c-raf* antisense oligonucleotide (ISIS 12854, SEQ ID NO. 2) on ERK, JNK, and p38 MAPK activities stimulated by TNF-α, *in vitro* kinase assays were performed on extracts derived from cells treated with ISIS 12854 (SEQ ID NO. 2). Cells were treated with oligonucleotides and induced with cytokines. At the indicated time, cells were lysed on ice and debris was removed by centrifugation. Protein concentration was measured by Bradford assay. Lysate containing equal amounts of protein were incubated with primary antibody-agarose conjugates (ERK and p38 assay; Santa Cruz Biotechnology, Santa Cruz, CA), or with JNK1-specific or JNK2-specific antibodies (JNK assay; Upstate Biotechnology, Lake Placid, NY), overnight at 4°C.

For isoform-specific JNK assays, anti-rabbit IgG conjugated with agarose beads was added to cell extracts following JNK antibody treatment and wash steps and incubated for 2 hours at 4°C. After washing with lysis buffer and kinase buffer, the pelleted beads were incubated with 1μg of substrate (Elk-1 for ERK, ATF-2 for p38, and c-Jun for JNK MAPK) and 100 αM of ATP for 20 minutes at 37°C. MAPK and JNK assay kits, ATF-2 fusion protein, and antibodies for ATF-2, Elk-1 phospho-ATF-2 were purchased from New England BioLabs (Boston, MA). Reactions were terminated by the addition of 3x SDS sample buffer followed by boiling. The samples were loaded on 12% SDS-PAGE gels. Western blot with antibodies specific for phosphorylated substrates (New England Biolabs) was carried out. The results were visualized by ECL.

Cells were treated with the *c-raf* antisense compound, ISIS 12854 (SEQ ID NO. 2), allowed to recover for 48 hours, at which time TNF-α was added for 5 or 15 minutes prior to cell lysis and initiation of the kinase assays. Specific antibody-conjugated agarose beads were used to immunoprecipitate ERK and p38 MAPK, and c-Jun-conjugated agarose beads were used to precipitate JNK. Suitable substrates and ATP were added to the immunoprecipitated kinase complexes and the reaction mixes were analyzed on SDS-PAGE. Western blotting with antibodies specific for phosphorylated substrates was carried out to determine relative kinase activity. Results are shown in Figure 3.

All three kinases were activated by TNF-α after a 15 minutes incubation, as indicated by the heavy phosphorylation of the three substrates. Inhibition of *c-raf* levels by ISIS 12854 (SEQ ID NO. 2) resulted in reduced ERK activity. Surprisingly, JNK activity was also inhibited by treating cells with ISIS 12854 (SEQ ID NO. 2). Activation of p38 MAPK was not affected by *c-raf* antisense treatment.

These results demonstrate for the first time that *c-raf* inhibition blocks TNF-α-mediated induction of cell adhesion molecules by suppressing the JNK pathway. Antisense oligonucleotides targeted against JNK1 (SEQ ID NO. 6) or JNK2 (SEQ ID NO. 7) were tested for their abilities to inhibit JNK expression, JNK activity and E-selectin induction by TNF-α. Oligonucleotide treatment, RNA isolation and Northern blots were performed as described in Example 2. A cDNA clone of JNK1 (Derijard *et al.*, *Cell*, 1994, *76*, 1025) was radiolabeled and used as a JNK1-specific probe. A cDNA clone of JNK2 (Kallunki *et al.*, *Genes & Development*, 1994, *8*, 2996) was radiolabeled and used as a JNK2-specific probe. JNK1 and JNK2 antisense treatment resulted in nearly complete inhibition of JNK1 and JNK2 mRNA expression, respectively, as shown in Figure 4. Furthermore, both antisense oligonucleotides were isoform-specific at the employed concentrations. The JNK2 antisense molecule will inhibit JNK1 expression slightly at higher oligonucleotide concentrations due to the fact that it is complementary to JNK1 mRNA in 17 of its 20 bases. However, at the concentration tested, the JNK2 antisense oligonucleotide specifically inhibits JNK2 expression without affecting JNK1 levels. Treatment of cells with either JNK1 or JNK2 antisense effectively reduced TNF-α-mediated induction of JNK activity in an isoform-specific manner, as shown in Figure 5. However, JNK2 antisense treatment resulted in substantially greater inhibition of TNF-α-mediated induction of E-selectin cell surface expression relative to JNK1 antisense treatment. Results are shown in Table 9. These results further confirm that the involvement of *c-raf* in TNF-α-mediated induction of cell adhesion molecules in HMVEC involves the regulation of JNK and that this regulation is believed to be specific for the JNK2 isoform.

TABLE 9

Dose Response of the Effect of JNK Antisense Oligonucleotides in induction of E-selectin

ISIS #	SEQ ID NO:	ASO Gene Target	Time (hours)	% Cell Surface Expression	% Cell Surface Inhibition
LIPOFECTIN	---	---	---	100%	---
15347	6	JNK1	20 nM	89%	11%
"	"	"	50 nM	84%	16%
"	"	"	100 nM	82%	18%
15354	7	JNK2	20 nM	32%	68%
"	"	"	50 nM	29%	71%
"	"	"	100 nM	33%	67%
15727	3	control	20 nM	137%	---
"	"	"	50 nM	128%	---
"	"	"	100 nM	124%	---

WHAT IS CLAIMED IS:

1. A method of modulating cell adhesion molecule expression comprising treating a cell expressing a cell adhesion molecule with a specific inhibitor of a Tumor Necrosis Factor alpha signaling molecule selected from the group consisting of Ha-*ras*, *c-raf* and JNK2, such that cell adhesion molecule expression is modulated.

2. The method of claim 1 wherein said cell adhesion molecule is E-selectin, VCAM-1 or ICAM-1.

3. The method of claim 1 wherein said inhibitor is an antisense oligonucleotide specifically hybridizable with a nucleic acid encoding Tumor Necrosis Factor alpha signaling molecule.

4. The method of claim 3 wherein said Tumor Necrosis Factor alpha signaling molecule is Ha-*ras* or *c-raf*.

5. The method of claim 4 wherein said antisense oligonucleotide is hybridizable to Ha-*ras* or *c-raf*.

6. The method of claim 5 wherein said antisense oligonucleotide has a sequence comprising SEQ ID NO. 2 or SEQ ID NO. 4.

7. The method of claim 6 wherein said antisense oligonucleotide has at least one phosphorothioate internucleotide linkage.

8. The method of claim 6 wherein said antisense oligonucleotide has at least one 2'-methoxyethoxy nucleotide.

9. The method of claim 1 wherein said cell adhesion molecule is E-selectin and said Tumor Necrosis Factor alpha signaling molecule is JNK-2.

10. The method of claim 9 wherein said inhibitor is an antisense oligonucleotide specifically hybridizable with a nucleic acid encoding JNK-2.

11. The method of claim 10 wherein said antisense oligonucleotide has a sequence comprising SEQ ID NO. 7.

12. The method of claim 11 wherein said antisense oligonucleotide has at least one phosphorothioate internucleotide linkage.

13. The method of claim 11 wherein said antisense oligonucleotide has at least one 2'-methoxyethoxy nucleotide.

14. A method of inhibiting expression of a MAP kinase within a cell comprising treating said cell with a specific inhibitor of *c-raf*.

15. The method of claim 14 wherein said inhibitor is an antisense oligonucleotide specifically hybridizable with a nucleic acid encoding *c-raf*.

16. The method of claim 15 wherein said oligonucleotide comprises SEQ ID NO. 2.

17. The method of claim 16 wherein said antisense oligonucleotide has at least one phosphorothioate internucleotide linkage.

18. The method of claim 16 wherein said antisense oligonucleotide has at least one 2'-methoxyethoxy nucleotide.

19. A method of treating a disease or condition associated with altered expression of a cell adhesion molecule comprising administering a specific inhibitor of a Tumor Necrosis Factor alpha signaling molecule selected from the group consisting of Ha-*ras*, *c-raf* and JNK2, under conditions wherein expression of said cell adhesion molecule is modulated.

20. The method of claim 19 wherein said disease or condition is an inflammatory or immune disease or condition.

21. The method of claim 19 wherein said disease is sepsis, rheumatoid arthritis,inflammatory bowel disease, allergic contact dermatitis, psoriasis, diabetes, Grave's disease, allograft rejection or cancer.

22. The method of claim 19 wherein said inhibitor is an antisense oligonucleotide specifically hybridizable with a nucleic acid encoding Tumor Necrosis Factor alpha signaling molecule.

23. The method of claim 22 wherein said antisense oligonucleotide has a sequence comprising SEQ ID NO. 2, SEQ ID NO. 4 or SEQ ID NO. 7.

24. The method of claim 23 wherein said antisense oligonucleotide has at least one phosphorothioate internucleotide linkage.

25. The method of claim 23 wherein said antisense oligonucleotide has at least one 2'-methoxyethoxy nucleotide.

26. An inhibitor of a Tumor Necrosis Factor alpha signaling molecule selected from the group consisting of Ha-*ras*, *c-raf* and JNK2, wherein said inhibitor modulates expression of a cell adhesion molecule.

27. The inhibitor of claim 26 wherein said cell adhesion molecule is E-selectin, VCAM-1 or ICAM-1.

28. The inhibitor of claim 26 comprising an antisense oligonucleotide specifically hybridizable with a nucleic acid encoding a Tumor Necrosis Factor alpha signaling molecule.

29. The inhibitor of claim 27 wherein said tumor Necrosis Facotr alpha signaling molecule is Ha-*ras* or *c-raf*.

30. The inhibitor of claim 28 wherein said antisense oligonucleotide is hybridizable to Ha-*ras* or *c-raf*.

31. The inhibitor of claim 30 wherein said antisense oligonucleotide has a sequence comprising SEQ ID NO. 2 or SEQ ID NO. 4.

32. The inhibitor of claim 31 wherein said antisense oligonucleotide has at least one phosphorothioate internucleotide linkage.

33. The inhibitor of claim 31 wherein said antisense oligonucleotide has at least one 2'-methoxyethoxy nucleotide.

34. The inhibitor of claim 26 wherein said cell adhesion molecule is E-selectin and said Tumor Necrosis Factor alpha signaling molecule is JNK-2.

35. The inhibitor of claim 34 comprising an antisense oligonucleotide specifically hybridizable with a nucleic acid encoding JNK-2.

36. The inhibitor of claim 35 wherein said antisense oligonucleotide has a sequence comprising SEQ ID NO. 7.

37. The inhibitor of claim 36 wherein said antisense oligonucleotide has at least one phosphorothioate internucleotide linkage.

38. The method of claim 36 wherein said antisense oligonucleotide has at least one 2'-methoxyethoxy nucleotide.

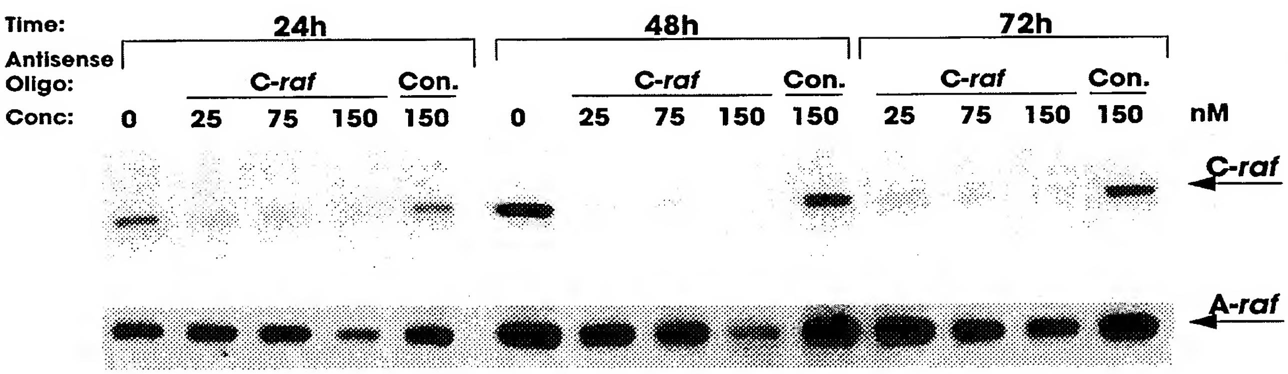
Time:
24h
48h
72h
Antisense Oligo:
C-raf
Con.
C-raf
Con.
C-raf
Con.
Conc:
0 25 75 150 150
0 25 75 150 150
25 75 150 150
nM
C-raf
A-raf

FIG. 1

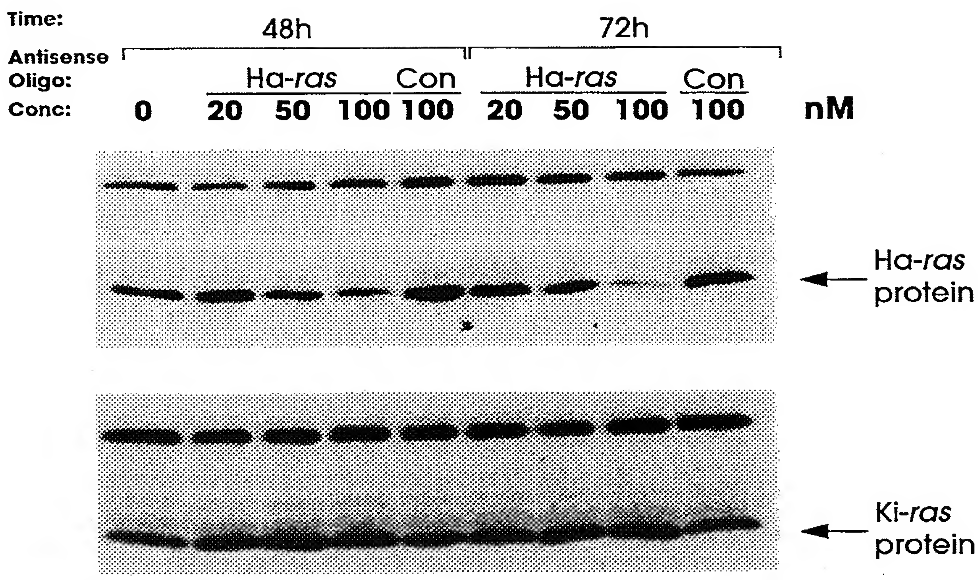
Time:
48h
72h
Antisense
Oligo:
Ha-ras
Con
Ha-ras
Con
Conc:
0
20
50
100
100
20
50
100
100
nM
Ha-ras protein
Ki-ras protein

FIG. 2

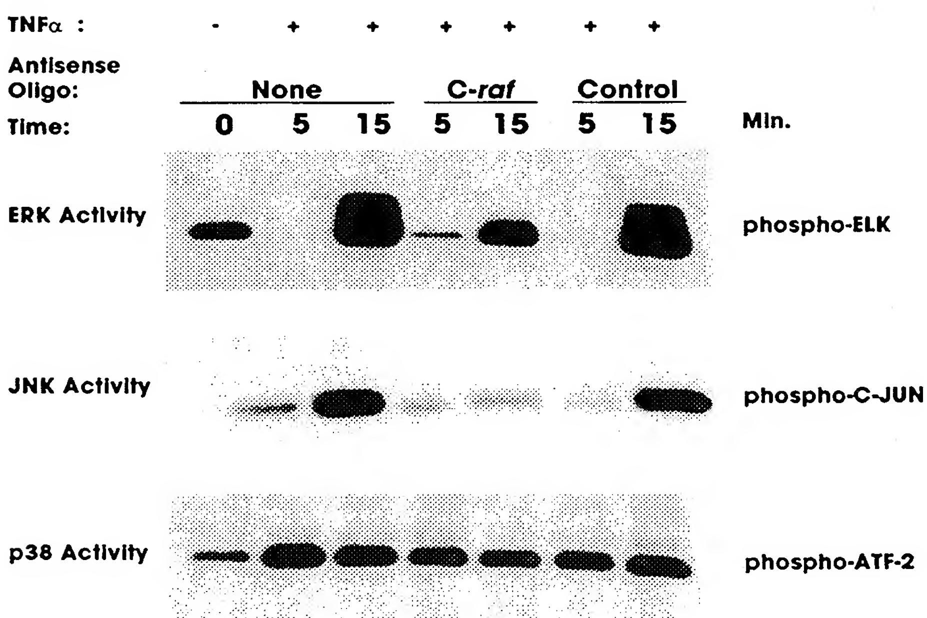
TNFα :
-
+
+
+
+
+
+
Antisense
Oligo:
None
C-raf
Control
Time:
0
5
15
5
15
5
15
Min.
ERK Activity
phospho-ELK
JNK Activity
phospho-C-JUN
p38 Activity
phospho-ATF-2

FIG. 3

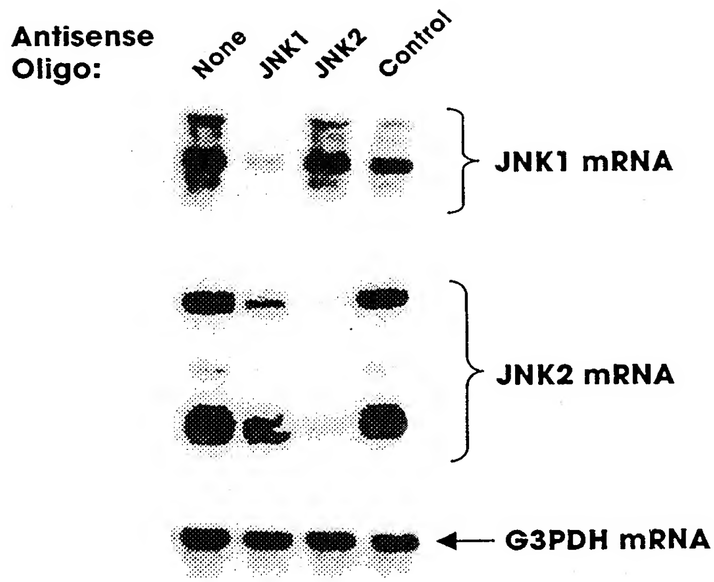
Antisense Oligo:
None
JNK1
JNK2
Control
JNK1 mRNA
JNK2 mRNA
G3PDH mRNA

FIG. 4

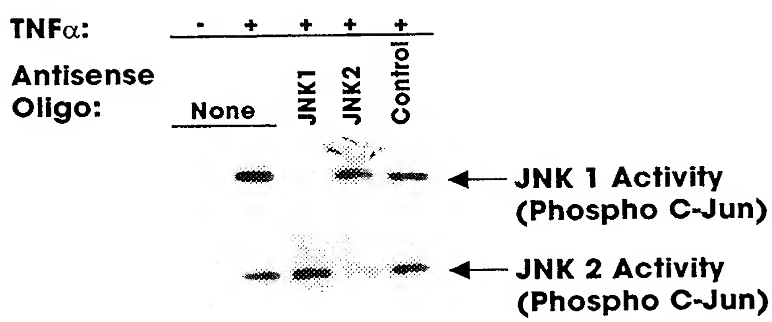
TNFα:
-
+
+
+
+
Antisense Oligo:
None
JNK1
JNK2
Control
JNK 1 Activity (Phospho C-Jun)
JNK 2 Activity (Phospho C-Jun)

FIG. 5

SEQUENCE LISTING

<110> Monia, Brett P.
Xu, Xiaoxing S.
Isis Pharmaceuticals, Inc.

<120> METHODS OF MODULATING TUMOR NECROSIS FACTOR alpha-INDUCED EXPRESSION OF CELL ADHESION MOLECULES

<130> ISPH-0424

<150> US 09/209,668
<151> 1998-12-10

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aagcaaagtt ttacaacttt ttaaagacat agactttatg ttattataat taaaaatcat 180

gcatttttgt catattaata aaattgcata tacgatataa aggcatggac aaaggtgaag 240

tagcttcaag agacagagtt tctgacatca ttgtaatttt aagcatcgtg gatattcccg 300

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                                              Met Ile Ala Ser Gln
                                               1               5

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Phe Leu Ser Ala Leu Thr Leu
                 10

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cggccctggc cccgggggca gtcgcgcctg tgaacggtga gtgcgggcag ggatcggccg 600

ggccgcgcgc cctcctcgcc cccaggcggc agcaatacgc gcggcgcggg ccgggggcgc 660

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Lys Leu Val Val Val Gly Ala Gly Gly Val Gly Lys Ser Ala Leu Thr
  5                  10                  15                  20

atc cag ctg atc cag aac cat ttt gtg gac gaa tac gac ccc act ata    1771
Ile Gln Leu Ile Gln Asn His Phe Val Asp Glu Tyr Asp Pro Thr Ile
                 25                  30                  35

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Glu

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                                                 40

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Val Val Ile Asp Gly Glu Thr Cys Leu Leu Asp Ile Leu Asp Thr Ala
     45                  50                  55

ggc cag gag gag tac agc gcc atg cgg gac cag tac atg cgc acc ggg    2155
Gly Gln Glu Glu Tyr Ser Ala Met Arg Asp Gln Tyr Met Arg Thr Gly
 60                  65                  70                  75

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Glu Gly Phe Leu Cys Val Phe Ala Ile Asn Asn Thr Lys Ser Phe Glu
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    Glu Gln Ile Lys Arg Val Lys Asp Ser Asp Asp Val Pro Met Val
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Leu Val Gly Asn Lys Cys Asp Leu Ala Ala Arg Thr Val Glu Ser Arg
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Gln Ala Gln Asp Leu Ala Arg Ser Tyr Gly Ile Pro Tyr Ile Glu Thr
    130                 135                 140

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50 55 60

Ser Ala Met Arg Asp Gln Tyr Met Arg Thr Gly Glu Gly Phe Leu Cys
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Val Phe Ala Ile Asn Asn Thr Lys Ser Phe Glu Asp Ile His Gln Tyr
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Gln Ala Gln Asp Leu Ala Arg Ser Tyr Gly Ile Pro Tyr Ile Glu Thr
130 135 140

Ser Ala Lys Thr Arg Gln Gly Val Glu Asp Ala Phe Tyr Thr Leu Val
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          Met Glu His Ile Gln Gly Ala Trp Lys Thr Ile Ser Asn Gly
           1               5                  10

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Phe Gly Phe Lys Asp Ala Val Phe Asp Gly Ser Ser Cys Ile Ser Pro
 15                  20                  25                  30

aca ata gtt cag cag ttt ggc tat cag cgc cgg gca tca gat gat ggc   267
Thr Ile Val Gln Gln Phe Gly Tyr Gln Arg Arg Ala Ser Asp Asp Gly
                35                  40                  45

aaa ctc aca gat cct tct aag aca agc aac act atc cgt gtt ttc ttg   315
Lys Leu Thr Asp Pro Ser Lys Thr Ser Asn Thr Ile Arg Val Phe Leu
            50                  55                  60

ccg aac aag caa aga aca gtg gtc aat gtg cga aat gga atg agc ttg   363
Pro Asn Lys Gln Arg Thr Val Val Asn Val Arg Asn Gly Met Ser Leu
        65                  70                  75

cat gac tgc ctt atg aaa gca ctc aag gtg agg ggc ctg caa cca gag   411
His Asp Cys Leu Met Lys Ala Leu Lys Val Arg Gly Leu Gln Pro Glu
    80                  85                  90

tgc tgt gca gtg ttc aga ctt ctc cac gaa cac aaa ggt aaa aaa gca   459
Cys Cys Ala Val Phe Arg Leu Leu His Glu His Lys Gly Lys Lys Ala
 95                 100                 105                 110

cgc tta gat tgg aat act gat gct gcg tct ttg att gga gaa gaa ctt   507
Arg Leu Asp Trp Asn Thr Asp Ala Ala Ser Leu Ile Gly Glu Glu Leu
                115                 120                 125

caa gta gat ttc ctg gat cat gtt ccc ctc aca aca cac aac ttt gct   555
Gln Val Asp Phe Leu Asp His Val Pro Leu Thr Thr His Asn Phe Ala
            130                 135                 140

cgg aag acg ttc ctg aag ctt gcc ttc tgt gac atc tgt cag aaa ttc   603
Arg Lys Thr Phe Leu Lys Leu Ala Phe Cys Asp Ile Cys Gln Lys Phe
        145                 150                 155

ctg ctc aat gga ttt cga tgt cag act tgt ggc tac aaa ttt cat gag   651
Leu Leu Asn Gly Phe Arg Cys Gln Thr Cys Gly Tyr Lys Phe His Glu
    160                 165                 170

cac tgt agc acc aaa gta cct act atg tgt gtg gac tgg agt aac atc   699
His Cys Ser Thr Lys Val Pro Thr Met Cys Val Asp Trp Ser Asn Ile
175                 180                 185                 190

aga caa ctc tta ttg ttt cca aat tcc act att ggt gat agt gga gtc   747
Arg Gln Leu Leu Leu Phe Pro Asn Ser Thr Ile Gly Asp Ser Gly Val
                195                 200                 205

cca gca cta cct tct ttg act atg cgt cgt atg cga gag tct gtt tcc   795
Pro Ala Leu Pro Ser Leu Thr Met Arg Arg Met Arg Glu Ser Val Ser
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Arg Met Pro Val Ser Ser Gln His Arg Tyr Ser Thr Pro His Ala Phe
        225                 230                 235

acc ttt aac acc tcc agt ccc tca tct gaa ggt tcc ctc tcc cag agg   891
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Gln Arg Ser Thr Ser Thr Pro Asn Val His Met Val Ser Thr Thr Leu
255                 260                 265                 270

cct gtg gac agc agg atg att gag gat gca att cga agt cac agc gaa      987
Pro Val Asp Ser Arg Met Ile Glu Asp Ala Ile Arg Ser His Ser Glu
                275                 280                 285

tca gcc tca cct tca gcc ctg tcc agt agc ccc aac aat ctg agc cca     1035
Ser Ala Ser Pro Ser Ala Leu Ser Ser Ser Pro Asn Asn Leu Ser Pro
            290                 295                 300

aca ggc tgg tca cag ccg aaa acc ccc gtg cca gca caa aga gag cgg     1083
Thr Gly Trp Ser Gln Pro Lys Thr Pro Val Pro Ala Gln Arg Glu Arg
        305                 310                 315

gca cca gta tct ggg acc cag gag aaa aac aaa att agg cct cgt gga     1131
Ala Pro Val Ser Gly Thr Gln Glu Lys Asn Lys Ile Arg Pro Arg Gly
    320                 325                 330

cag aga gat tca agc tat tat tgg gaa ata gaa gcc agt gaa gtg atg     1179
Gln Arg Asp Ser Ser Tyr Tyr Trp Glu Ile Glu Ala Ser Glu Val Met
335                 340                 345                 350

ctg tcc act cgg att ggg tca ggc tct ttt gga act gtt tat aag ggt     1227
Leu Ser Thr Arg Ile Gly Ser Gly Ser Phe Gly Thr Val Tyr Lys Gly
                355                 360                 365

aaa tgg cac gga gat gtt gca gta aag atc cta aag gtt gtc gac cca     1275
Lys Trp His Gly Asp Val Ala Val Lys Ile Leu Lys Val Val Asp Pro
            370                 375                 380

acc cca gag caa ttc cag gcc ttc agg aat gag gtg gct gtt ctg cgc     1323
Thr Pro Glu Gln Phe Gln Ala Phe Arg Asn Glu Val Ala Val Leu Arg
        385                 390                 395

aaa aca cgg cat gtg aac att ctg ctt ttc atg ggg tac atg aca aag     1371
Lys Thr Arg His Val Asn Ile Leu Leu Phe Met Gly Tyr Met Thr Lys
    400                 405                 410

gac aac ctg gca att gtg acc cag tgg tgc gag ggc agc agc ctc tac     1419
Asp Asn Leu Ala Ile Val Thr Gln Trp Cys Glu Gly Ser Ser Leu Tyr
415                 420                 425                 430

aaa cac ctg cat gtc cag gag acc aag ttt cag atg ttc cag cta att     1467
Lys His Leu His Val Gln Glu Thr Lys Phe Gln Met Phe Gln Leu Ile
                435                 440                 445

gac att gcc cgg cag acg gct cag gga atg gac tat ttg cat gca aag     1515
Asp Ile Ala Arg Gln Thr Ala Gln Gly Met Asp Tyr Leu His Ala Lys
            450                 455                 460

aac atc atc cat aga gac atg aaa tcc aac aat ata ttt ctc cat gaa     1563
Asn Ile Ile His Arg Asp Met Lys Ser Asn Asn Ile Phe Leu His Glu
        465                 470                 475

ggc tta aca gtg aaa att gga gat ttt ggt ttg gca aca gta aag tca     1611
Gly Leu Thr Val Lys Ile Gly Asp Phe Gly Leu Ala Thr Val Lys Ser
    480                 485                 490
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cgc tgg agt ggt tct cag cag gtt gaa caa cct act ggc tct gtc ctc    1659
Arg Trp Ser Gly Ser Gln Gln Val Glu Gln Pro Thr Gly Ser Val Leu
495                 500                 505                 510

tgg atg gcc cca gag gtg atc cga atg cag gat aac aac cca ttc agt    1707
Trp Met Ala Pro Glu Val Ile Arg Met Gln Asp Asn Asn Pro Phe Ser
                515                 520                 525

ttc cag tcg gat gtc tac tcc tat ggc atc gta ttg tat gaa ctg atg    1755
Phe Gln Ser Asp Val Tyr Ser Tyr Gly Ile Val Leu Tyr Glu Leu Met
            530                 535                 540

acg ggg gag ctt cct tat tct cac atc aac aac cga gat cag atc atc    1803
Thr Gly Glu Leu Pro Tyr Ser His Ile Asn Asn Arg Asp Gln Ile Ile
        545                 550                 555

ttc atg gtg ggc cga gga tat gcc tcc cca gat ctt agt aag cta tat    1851
Phe Met Val Gly Arg Gly Tyr Ala Ser Pro Asp Leu Ser Lys Leu Tyr
    560                 565                 570

aag aac tgc ccc aaa gca atg aag agg ctg gta gct gac tgt gtg aag    1899
Lys Asn Cys Pro Lys Ala Met Lys Arg Leu Val Ala Asp Cys Val Lys
575                 580                 585                 590

aaa gta aag gaa gag agg cct ctt ttt ccc cag atc ctg tct tcc att    1947
Lys Val Lys Glu Glu Arg Pro Leu Phe Pro Gln Ile Leu Ser Ser Ile
                595                 600                 605

gag ctg ctc caa cac tct cta ccg aag atc aac cgg agc gct tcc gag    1995
Glu Leu Leu Gln His Ser Leu Pro Lys Ile Asn Arg Ser Ala Ser Glu
            610                 615                 620

cca tcc ttg cat cgg gca gcc cac act gag gat atc aat gct tgc acg    2043
Pro Ser Leu His Arg Ala Ala His Thr Glu Asp Ile Asn Ala Cys Thr
        625                 630                 635

ctg acc acg tcc ccg agg ctg cct gtc ttc tag ttgactttgc acctgtcttc 2096
Leu Thr Thr Ser Pro Arg Leu Pro Val Phe
    640                 645

aggctgccag gggaggagga gaagccagca ggcaccactt ttctgctccc tttctccaga 2156

ggcagaacac atgttttcag agaagctctg ctaaggacct tctagactgc tcacagggcc 2216

ttaacttcat gttgccttct tttctatccc tttgggccct gggagaagga agccatttgc 2276

agtgctggtg tgtcctgctc cctccccaca ttccccatgc tcaaggccca gccttctgta 2336

gatgcgcaag tggatgttga tggtagtaca aaaagcaggg gcccagcccc agctgttggc 2396

tacatgagta tttagaggaa gtaaggtagc aggcagtcca gccctgatgt ggagacacat 2456

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gagctgcccc tatggggcgg gccgcagggc cagcctgttt ctctaacaaa caaacaaaca 2816
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aacagccttg tttctctagt cacatcatgt gtatacaagg aagccaggaa tacaggtttt 2876

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ggtccctcaa ttatgttatt ttaataaaat aaattaaatt t 2977

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Val Gln Gln Phe Gly Tyr Gln Arg Arg Ala Ser Asp Asp Gly Lys Leu
35 40 45

Thr Asp Pro Ser Lys Thr Ser Asn Thr Ile Arg Val Phe Leu Pro Asn
50 55 60

Lys Gln Arg Thr Val Val Asn Val Arg Asn Gly Met Ser Leu His Asp
65 70 75 80

Cys Leu Met Lys Ala Leu Lys Val Arg Gly Leu Gln Pro Glu Cys Cys
85 90 95

Ala Val Phe Arg Leu Leu His Glu His Lys Gly Lys Lys Ala Arg Leu
100 105 110

Asp Trp Asn Thr Asp Ala Ala Ser Leu Ile Gly Glu Glu Leu Gln Val
115 120 125

Asp Phe Leu Asp His Val Pro Leu Thr Thr His Asn Phe Ala Arg Lys
130 135 140

Thr Phe Leu Lys Leu Ala Phe Cys Asp Ile Cys Gln Lys Phe Leu Leu
145 150 155 160

Asn Gly Phe Arg Cys Gln Thr Cys Gly Tyr Lys Phe His Glu His Cys
165 170 175

Ser Thr Lys Val Pro Thr Met Cys Val Asp Trp Ser Asn Ile Arg Gln
180 185 190

Leu Leu Leu Phe Pro Asn Ser Thr Ile Gly Asp Ser Gly Val Pro Ala
195 200 205

Leu Pro Ser Leu Thr Met Arg Arg Met Arg Glu Ser Val Ser Arg Met
210 215 220

Pro Val Ser Ser Gln His Arg Tyr Ser Thr Pro His Ala Phe Thr Phe
225 230 235 240

Asn Thr Ser Ser Pro Ser Ser Glu Gly Ser Leu Ser Gln Arg Gln Arg
245 250 255

Ser Thr Ser Thr Pro Asn Val His Met Val Ser Thr Thr Leu Pro Val
260 265 270

Asp Ser Arg Met Ile Glu Asp Ala Ile Arg Ser His Ser Glu Ser Ala
275 280 285

Ser Pro Ser Ala Leu Ser Ser Ser Pro Asn Asn Leu Ser Pro Thr Gly
290 295 300

Trp Ser Gln Pro Lys Thr Pro Val Pro Ala Gln Arg Glu Arg Ala Pro
305 310 315 320

Val Ser Gly Thr Gln Glu Lys Asn Lys Ile Arg Pro Arg Gly Gln Arg
325 330 335

Asp Ser Ser Tyr Tyr Trp Glu Ile Glu Ala Ser Glu Val Met Leu Ser
340 345 350

Thr Arg Ile Gly Ser Gly Ser Phe Gly Thr Val Tyr Lys Gly Lys Trp
355 360 365

His Gly Asp Val Ala Val Lys Ile Leu Lys Val Val Asp Pro Thr Pro
370 375 380

Glu Gln Phe Gln Ala Phe Arg Asn Glu Val Ala Val Leu Arg Lys Thr
385 390 395 400

Arg His Val Asn Ile Leu Leu Phe Met Gly Tyr Met Thr Lys Asp Asn
405 410 415

Leu Ala Ile Val Thr Gln Trp Cys Glu Gly Ser Ser Leu Tyr Lys His
420 425 430

Leu His Val Gln Glu Thr Lys Phe Gln Met Phe Gln Leu Ile Asp Ile
435 440 445

Ala Arg Gln Thr Ala Gln Gly Met Asp Tyr Leu His Ala Lys Asn Ile
450 455 460

Ile His Arg Asp Met Lys Ser Asn Asn Ile Phe Leu His Glu Gly Leu
465 470 475 480

Thr Val Lys Ile Gly Asp Phe Gly Leu Ala Thr Val Lys Ser Arg Trp
485 490 495

Ser Gly Ser Gln Gln Val Glu Gln Pro Thr Gly Ser Val Leu Trp Met
500 505 510

Ala Pro Glu Val Ile Arg Met Gln Asp Asn Asn Pro Phe Ser Phe Gln
515 520 525

Ser Asp Val Tyr Ser Tyr Gly Ile Val Leu Tyr Glu Leu Met Thr Gly
530 535 540

Glu Leu Pro Tyr Ser His Ile Asn Asn Arg Asp Gln Ile Ile Phe Met
545 550 555 560

Val Gly Arg Gly Tyr Ala Ser Pro Asp Leu Ser Lys Leu Tyr Lys Asn
565 570 575

Cys Pro Lys Ala Met Lys Arg Leu Val Ala Asp Cys Val Lys Lys Val
580 585 590

Lys Glu Glu Arg Pro Leu Phe Pro Gln Ile Leu Ser Ser Ile Glu Leu
595 600 605

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Leu Gln His Ser Leu Pro Lys Ile Asn Arg Ser Ala Ser Glu Pro Ser
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Leu His Arg Ala Ala His Thr Glu Asp Ile Asn Ala Cys Thr Leu Thr
625                 630                 635                 640

Thr Ser Pro Arg Leu Pro Val Phe
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                     1               5                  10

agt gta gag att gga gat tct aca ttc aca gtc ctg aaa cga tat cag      99
Ser Val Glu Ile Gly Asp Ser Thr Phe Thr Val Leu Lys Arg Tyr Gln
            15                  20                  25

aat tta aaa cct ata ggc tca gga gct caa gga ata gta tgc gca gct     147
Asn Leu Lys Pro Ile Gly Ser Gly Ala Gln Gly Ile Val Cys Ala Ala
        30                  35                  40

tat gat gcc att ctt gaa aga aat gtt gca atc aag aag cta agc cga     195
Tyr Asp Ala Ile Leu Glu Arg Asn Val Ala Ile Lys Lys Leu Ser Arg
    45                  50                  55

cca ttt cag aat cag act cat gcc aag cgg gcc tac aga gag cta gtt     243
Pro Phe Gln Asn Gln Thr His Ala Lys Arg Ala Tyr Arg Glu Leu Val
60                  65                  70                  75

ctt atg aaa tgt gtt aat cac aaa aat ata att ggc ctt ttg aat gtt     291
Leu Met Lys Cys Val Asn His Lys Asn Ile Ile Gly Leu Leu Asn Val
                80                  85                  90

ttc aca cca cag aaa tcc cta gaa gaa ttt caa gat gtt tac ata gtc     339
Phe Thr Pro Gln Lys Ser Leu Glu Glu Phe Gln Asp Val Tyr Ile Val
            95                  100                 105

atg gag ctc atg gat gca aat ctt tgc caa gtg att cag atg gag cta     387
Met Glu Leu Met Asp Ala Asn Leu Cys Gln Val Ile Gln Met Glu Leu
        110                 115                 120

gat cat gaa aga atg tcc tac ctt ctc tat cag atg ctg tgt gga atc     435
Asp His Glu Arg Met Ser Tyr Leu Leu Tyr Gln Met Leu Cys Gly Ile
    125                 130                 135

aag cac ctt cat tct gct gga att att cat cgg gac tta aag ccc agt     483
Lys His Leu His Ser Ala Gly Ile Ile His Arg Asp Leu Lys Pro Ser
140                 145                 150                 155
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aat ata gta gta aaa tct gat tgc act ttg aag att ctt gac ttc ggt    531
Asn Ile Val Val Lys Ser Asp Cys Thr Leu Lys Ile Leu Asp Phe Gly
                160                 165                 170

ctg gcc agg act gca gga acg agt ttt atg atg acg cct tat gta gtg    579
Leu Ala Arg Thr Ala Gly Thr Ser Phe Met Met Thr Pro Tyr Val Val
            175                 180                 185

act cgc tac tac aga gca ccc gag gtc atc ctt ggc atg ggc tac aag    627
Thr Arg Tyr Tyr Arg Ala Pro Glu Val Ile Leu Gly Met Gly Tyr Lys
        190                 195                 200

gaa aac gtg gat tta tgg tct gtg ggg tgc att atg gga gaa atg gtt    675
Glu Asn Val Asp Leu Trp Ser Val Gly Cys Ile Met Gly Glu Met Val
    205                 210                 215

tgc cac aaa atc ctc ttt cca gga agg gac tat att gat cag tgg aat    723
Cys His Lys Ile Leu Phe Pro Gly Arg Asp Tyr Ile Asp Gln Trp Asn
220                 225                 230                 235

aaa gtt att gaa cag ctt gga aca cca tgt cct gaa ttc atg aag aaa    771
Lys Val Ile Glu Gln Leu Gly Thr Pro Cys Pro Glu Phe Met Lys Lys
                240                 245                 250

ctg caa cca aca gta agg act tac gtt gaa aac aga cct aaa tat gct    819
Leu Gln Pro Thr Val Arg Thr Tyr Val Glu Asn Arg Pro Lys Tyr Ala
            255                 260                 265

gga tat agc ttt gag aaa ctc ttc cct gat gtc ctt ttc cca gct gac    867
Gly Tyr Ser Phe Glu Lys Leu Phe Pro Asp Val Leu Phe Pro Ala Asp
        270                 275                 280

tca gaa cac aac aaa ctt aaa gcc agt cag gca agg gat ttg tta tcc    915
Ser Glu His Asn Lys Leu Lys Ala Ser Gln Ala Arg Asp Leu Leu Ser
    285                 290                 295

aaa atg ctg gta ata gat gca tct aaa agg atc tct gta gat gaa gct    963
Lys Met Leu Val Ile Asp Ala Ser Lys Arg Ile Ser Val Asp Glu Ala
300                 305                 310                 315

ctc caa cac ccg tac atc aat gtc tgg tat gat cct tct gaa gca gaa    1011
Leu Gln His Pro Tyr Ile Asn Val Trp Tyr Asp Pro Ser Glu Ala Glu
                320                 325                 330

gct cca cca cca aag atc cct gac aag cag tta gat gaa agg gaa cac    1059
Ala Pro Pro Pro Lys Ile Pro Asp Lys Gln Leu Asp Glu Arg Glu His
            335                 340                 345

aca ata gaa gag tgg aaa gaa ttg ata tat aag gaa gtt atg gac ttg    1107
Thr Ile Glu Glu Trp Lys Glu Leu Ile Tyr Lys Glu Val Met Asp Leu
        350                 355                 360

gag gag aga acc aag aat gga gtt ata cgg ggg cag ccc tct cct tta    1155
Glu Glu Arg Thr Lys Asn Gly Val Ile Arg Gly Gln Pro Ser Pro Leu
    365                 370                 375

gca cag gtg cag cag tga tcaatggctc tcagcatcca tcatcatcgt           1203
Ala Gln Val Gln Gln
380                 385

cgtctgtcaa tgatgtgtct tcaatgtcaa cagatccgac tttggcctct gatacagaca 1263

gcagtctaga agcagcagct gggcctctgg gctgctgtag atgactactt gggccatcgg 1323
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35 40 45

Glu Arg Asn Val Ala Ile Lys Lys Leu Ser Arg Pro Phe Gln Asn Gln
50 55 60

Thr His Ala Lys Arg Ala Tyr Arg Glu Leu Val Leu Met Lys Cys Val
65 70 75 80

Asn His Lys Asn Ile Ile Gly Leu Leu Asn Val Phe Thr Pro Gln Lys
85 90 95

Ser Leu Glu Glu Phe Gln Asp Val Tyr Ile Val Met Glu Leu Met Asp
100 105 110

Ala Asn Leu Cys Gln Val Ile Gln Met Glu Leu Asp His Glu Arg Met
115 120 125

Ser Tyr Leu Leu Tyr Gln Met Leu Cys Gly Ile Lys His Leu His Ser
130 135 140

Ala Gly Ile Ile His Arg Asp Leu Lys Pro Ser Asn Ile Val Val Lys
145 150 155 160

Ser Asp Cys Thr Leu Lys Ile Leu Asp Phe Gly Leu Ala Arg Thr Ala
165 170 175

Gly Thr Ser Phe Met Met Thr Pro Tyr Val Val Thr Arg Tyr Tyr Arg
180 185 190

Ala Pro Glu Val Ile Leu Gly Met Gly Tyr Lys Glu Asn Val Asp Leu
195 200 205

Trp Ser Val Gly Cys Ile Met Gly Glu Met Val Cys His Lys Ile Leu
210 215 220

Phe Pro Gly Arg Asp Tyr Ile Asp Gln Trp Asn Lys Val Ile Glu Gln
225 230 235 240

Leu Gly Thr Pro Cys Pro Glu Phe Met Lys Lys Leu Gln Pro Thr Val
245 250 255

Arg Thr Tyr Val Glu Asn Arg Pro Lys Tyr Ala Gly Tyr Ser Phe Glu
260 265 270

Lys Leu Phe Pro Asp Val Leu Phe Pro Ala Asp Ser Glu His Asn Lys

275 280 285

Leu Lys Ala Ser Gln Ala Arg Asp Leu Leu Ser Lys Met Leu Val Ile
290 295 300

Asp Ala Ser Lys Arg Ile Ser Val Asp Glu Ala Leu Gln His Pro Tyr
305 310 315 320

Ile Asn Val Trp Tyr Asp Pro Ser Glu Ala Glu Ala Pro Pro Pro Lys
325 330 335

Ile Pro Asp Lys Gln Leu Asp Glu Arg Glu His Thr Ile Glu Glu Trp
340 345 350

Lys Glu Leu Ile Tyr Lys Glu Val Met Asp Leu Glu Glu Arg Thr Lys
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Met Ser Asp Ser Lys Cys Asp Ser Gln Phe Tyr Ser Val Gln Val Ala
1 5 10 15

gac tca acc ttc act gtc cta aaa cgt tac cag cag ctg aaa cca att 154
Asp Ser Thr Phe Thr Val Leu Lys Arg Tyr Gln Gln Leu Lys Pro Ile
20 25 30

ggc tct ggg gcc caa ggg att gtt tgt gct gca ttt gat aca gtt ctt 202
Gly Ser Gly Ala Gln Gly Ile Val Cys Ala Ala Phe Asp Thr Val Leu
35 40 45

ggg ata agt gtt gca gtc aag aaa cta agc cgt cct ttt cag aac caa 250
Gly Ile Ser Val Ala Val Lys Lys Leu Ser Arg Pro Phe Gln Asn Gln
50 55 60

act cat gca aag aga gct tat cgt gaa ctt gtc ctc tta aaa tgt gtc 298
Thr His Ala Lys Arg Ala Tyr Arg Glu Leu Val Leu Leu Lys Cys Val
65 70 75 80

aat cat aaa aat ata att agt ttg tta aat gtg ttt aca cca caa aaa 346
Asn His Lys Asn Ile Ile Ser Leu Leu Asn Val Phe Thr Pro Gln Lys
85 90 95

act cta gaa gaa ttt caa gat gtg tat ttg gtt atg gaa tta atg gat 394
Thr Leu Glu Glu Phe Gln Asp Val Tyr Leu Val Met Glu Leu Met Asp

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          100                 105                 110

gct aac tta tgt cag gtt att cac atg gag ctg gat cat gaa aga atg    442
Ala Asn Leu Cys Gln Val Ile His Met Glu Leu Asp His Glu Arg Met
        115                 120                 125

tcc tac ctt ctt tac cag atg ctt tgt ggt att aaa cat ctg cat tca    490
Ser Tyr Leu Leu Tyr Gln Met Leu Cys Gly Ile Lys His Leu His Ser
    130                 135                 140

gct ggt ata att cat aga gat ttg aag cct agc aac att gtt gtg aaa    538
Ala Gly Ile Ile His Arg Asp Leu Lys Pro Ser Asn Ile Val Val Lys
145                 150                 155                 160

tca gac tgc acc ctg aag atc ctt gac ttt ggc ctg gcc cgg aca gcg    586
Ser Asp Cys Thr Leu Lys Ile Leu Asp Phe Gly Leu Ala Arg Thr Ala
                165                 170                 175

tgc act aac ttc atg atg acc cct tac gtg gtg aca cgg tac tac cgg    634
Cys Thr Asn Phe Met Met Thr Pro Tyr Val Val Thr Arg Tyr Tyr Arg
            180                 185                 190

gcg ccc gaa gtc atc ctg ggt atg ggc tac aaa gag aac gtt gat atc    682
Ala Pro Glu Val Ile Leu Gly Met Gly Tyr Lys Glu Asn Val Asp Ile
        195                 200                 205

tgg tca gtg ggt tgc atc atg gga gag ctg gtg aaa ggt tgt gtg ata    730
Trp Ser Val Gly Cys Ile Met Gly Glu Leu Val Lys Gly Cys Val Ile
    210                 215                 220

ttc caa ggc act gac cat att gat cag tgg aat aaa gtt att gag cag    778
Phe Gln Gly Thr Asp His Ile Asp Gln Trp Asn Lys Val Ile Glu Gln
225                 230                 235                 240

ctg gga aca cca tca gca gag ttc atg aag aaa ctt cag cca act gtg    826
Leu Gly Thr Pro Ser Ala Glu Phe Met Lys Lys Leu Gln Pro Thr Val
                245                 250                 255

agg aat tat gtc gaa aac aga cca aag tat cct gga atc aaa ttt gaa    874
Arg Asn Tyr Val Glu Asn Arg Pro Lys Tyr Pro Gly Ile Lys Phe Glu
            260                 265                 270

gaa ctc ttt cca gat tgg ata ttc cca tca gaa tct gag cga gac aaa    922
Glu Leu Phe Pro Asp Trp Ile Phe Pro Ser Glu Ser Glu Arg Asp Lys
        275                 280                 285

ata aaa aca agt caa gcc aga gat ctg tta tca aaa atg tta gtg att    970
Ile Lys Thr Ser Gln Ala Arg Asp Leu Leu Ser Lys Met Leu Val Ile
    290                 295                 300

gat cct gac aag cgg atc tct gta gac gaa gct ctg cgt cac cca tac    1018
Asp Pro Asp Lys Arg Ile Ser Val Asp Glu Ala Leu Arg His Pro Tyr
305                 310                 315                 320

atc act gtt tgg tat gac ccc gcc gaa gca gaa gcc cca cca cct caa    1066
Ile Thr Val Trp Tyr Asp Pro Ala Glu Ala Glu Ala Pro Pro Pro Gln
                325                 330                 335

att tat gat gcc cag ttg gaa gaa aga gaa cat gca att gaa gaa tgg    1114
Ile Tyr Asp Ala Gln Leu Glu Glu Arg Glu His Ala Ile Glu Glu Trp
            340                 345                 350

aaa gag cta att tac aaa gaa gtc atg gat tgg gaa gaa aga agc aag    1162
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Lys Glu Leu Ile Tyr Lys Glu Val Met Asp Trp Glu Glu Arg Ser Lys
355 360 365

aat ggt gtt gta aaa gat cag cct tca gat gca gca gta agt agc aac 1210
Asn Gly Val Val Lys Asp Gln Pro Ser Asp Ala Ala Val Ser Ser Asn
370 375 380

gcc act cct tct cag tct tca tcg atc aat gac att tca tcc atg tcc 1258
Ala Thr Pro Ser Gln Ser Ser Ser Ile Asn Asp Ile Ser Ser Met Ser
385 390 395 400

act gag cag acg ctg gcc tca gac aca gac agc agt ctt gat gcc tcg 1306
Thr Glu Gln Thr Leu Ala Ser Asp Thr Asp Ser Ser Leu Asp Ala Ser
405 410 415

acg gga ccc ctt gaa ggc tgt cga tga taggttagaa atagcaaacc 1353
Thr Gly Pro Leu Glu Gly Cys Arg
420 425

tgtcagcatt gaaggaactc tcacctccgt gggcctgaaa tgcttgggag ttgatggaac 1413

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gtaatttgg 1782

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Gly Ser Gly Ala Gln Gly Ile Val Cys Ala Ala Phe Asp Thr Val Leu
35 40 45

Gly Ile Ser Val Ala Val Lys Lys Leu Ser Arg Pro Phe Gln Asn Gln
50 55 60

Thr His Ala Lys Arg Ala Tyr Arg Glu Leu Val Leu Leu Lys Cys Val
65 70 75 80

Asn His Lys Asn Ile Ile Ser Leu Leu Asn Val Phe Thr Pro Gln Lys
85 90 95

Thr Leu Glu Glu Phe Gln Asp Val Tyr Leu Val Met Glu Leu Met Asp
100 105 110

Ala Asn Leu Cys Gln Val Ile His Met Glu Leu Asp His Glu Arg Met
115 120 125

Ser Tyr Leu Leu Tyr Gln Met Leu Cys Gly Ile Lys His Leu His Ser
130 135 140

Ala Gly Ile Ile His Arg Asp Leu Lys Pro Ser Asn Ile Val Val Lys
145 150 155 160

Ser Asp Cys Thr Leu Lys Ile Leu Asp Phe Gly Leu Ala Arg Thr Ala
165 170 175

Cys Thr Asn Phe Met Met Thr Pro Tyr Val Val Thr Arg Tyr Tyr Arg
180 185 190

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/28965

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : CO7H 21/04, 21/02; C12Q 1/68; A61K 48/00
US CL : 536/ 23.1, 24.3, 24.31, 24.33, 24.5; 435/6, 91.1; 514/44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 536/ 23.1, 24.3, 24.31, 24.33, 24.5; 435/6, 91.1; 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 5,656,612 A (MONIA et al) 12 August 1997 (12.08.1997), column 17, line 19.	26-33 -------- 14-18
X --- Y	WO 92-22651 A1 (ISIS PHARMACEUTICALS, INC.) 23 December 1992 (23.12.92), page 15, lines 1-8.	26-33 -------- 14-18
X,P --- Y,P	US 5,877,309 A (MCKAY et al) 02 March 1999 (02.03.99), col. 43.	34-38 ------------------------ 1-3, 9-13, 26-28
A	MIN W. et al TNF Initiates E-selectin Transcription in Human Endothelial Cell Through Parallel TRAF-NF-kB and TRAF-RAC/CDC42-JNK-c-Jun/ATF2 Pathways Journal of Immunology. July 1997, Vol. 159. No. 7, pages 3508-3518, especially page 3515.	1-3, 9-13, 26-28, 34-38
X	US 5,405,941 A (JOHNSON) 11 April 1995 (11.04.95), column 12, lines 18-34.	14-15

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 February 2000 (15.02.2000)	08 MAR 2000

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	George Elliott
Facsimile No. (703)305-3230	Telephone No. 703-308-0196

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/28965

Continuation of B. FIELDS SEARCHED Item 3: USPAT, EPO, JPO, Derwent, Caplus, Registry
search terms: antisense, aptamer, triplex, ribozyme, oligonucleotide, TNF-a, "tumor necrosis factor", cell adhesion, ICAM-1, E-selectin, VCAM-1.